Future vulnerability of *Modiolus modiolus* reefs to climate change: From mechanisms to management

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Doctor of Philosophy

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Abstract

This project was developed to address management needs for *Modiolus modiolus* reefs across the UK under the context of climate change. Investigations focused on reef populations at the southern limit of the range and northward over a latitudinal gradient including sites in Wales and the Isle of Man, off the coast of mainland Scotland and around Orkney and Shetland Islands. To improve understanding of species- and population-level vulnerabilities to warming and hypoxia, three key influences were studied: (1) environmental exposure, via collation of data on historical and future site conditions; (2) adaptive capacity, via determination of population genetic structure and connectivity; and (3) acclimatory ability, via investigation of oxidative stress response and energetic demands under climate change conditions. Results suggest that *M. modiolus* is vulnerable to future warming and hypoxia. However, vulnerability varies according to site conditions, stressor type, and exposure duration, and factors like feed availability and demographics may influence response. Consequently, it is concluded that populations face considerably different levels of climate change threat. Additionally, given moderate to high levels of connectivity between populations, adaptive capacity is likely to be low. Results are discussed collectively and implications for management, including contribution of evidence to management tools, are considered.

Acknowledgements

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PhD Project Outputs

Peer-reviewed

Brash JM, Cook RL, **Mackenzie CL**, Sanderson WG (2017) The demographics and morphometries of biogenic reefs: important considerations in conservation management. *Journal of the Marine Biological Association of the United Kingdom*: 1-10

Whiteley NM, **Mackenzie CL** (2016) Physiological responses of marine invertebrates to thermal stress. In: Solan, M and Whiteley, MN. Eds. *Stressors in the marine environment: physiological and ecological responses and societal implications*. Oxford University Press. pp. 56-72.

Gormley KSG, **Mackenzie CL**, Robins R, Coscia I, Cassidy AJ, James J, Hull AD, Piertney S, Sanderson WG, Porter JS (2015) Connectivity and dispersal patterns of protected biogenic reefs: implications for the conservation of *Modiolus modiolus* (L.) in the Irish Sea. *PloS ONE* 10(12): e0143337.

Reports

Mackenzie CL, Grieve RC, Porter JS (2017) Assessment of Little Ness (Isle of Man) Horse Mussel Reef: Survey Data, Genetic Analyses, Stress Response. Commissioned by Isle of Man Government. 35 pp.

Mackenzie CL, Kent F, Baxter JM, Porter JS (2017) Genetic analysis of horse mussel bed populations in Scotland. Commissioned by Scottish Natural Heritage.

Mackenzie CL (2016) An overview of Regional MPA Management Plans including a template Regional MPA Management Plan for the West Highlands Marine Region. Completed for Scottish Natural Heritage as part of internship placement (supervised by Sarah Cunningham).

Gormley KSG, **Mackenzie** CL, Porter JS (2014) Microsatellite genetic screening of *Modiolus modiolus* from Strangford Lough and Ards Peninsula, Northern Ireland. Commissioned by Northern Ireland Environment Agency.

Table of Contents

1. Introduction

1.1 Background and Context

Climate Change

Current global levels of atmospheric carbon dioxide ($CO_{2(atm)}$) are the highest experienced in 800,000 years and have increased by approximately 30% over the past 250 years (Sabine *et al.*, 2004; Guinotte & Fabry, 2008; Doney *et al.*, 2009; IPCC, 2013). Present $CO_{2(atm)}$ concentrations are now more than 400 ppmv (parts per million by volume) (Figure 1.1), increased from pre-industrial levels of approximately 280 ppmv (, and are continuing to escalate at a rate of increase approximately one order magnitude higher than has occurred for millions of years (Guinotte & Fabry, 2008; Doney *et al.*, 2009). Moreover, models predict current levels to continue increasing by 0.5% per year, with projections double to triple that of present-day amounts by the end of the century (Doney, 2006; Guinotte & Fabry, 2008; IPCC, 2013). These increases are chiefly attributed to anthropogenic activities including the combustion of fossil fuels such as oil, coal and natural gas as well as deforestation, industrialization and cement production (Guinotte & Fabry, 2008; IPCC, 2013).

Though a large amount of CO_2 derived from human activities remains in the atmosphere, the oceans constitute a considerable sink, accounting for at least one third of all anthropogenic carbon (Sabine *et al.*, 2004; Doney, 2006; IPCC, 2013). Increasing CO_2 concentrations in both the atmosphere and ocean have implications for marine environments. Firstly, increased levels of CO_2 in the atmosphere are responsible for global warming of seawater. Hydrographic surveys have confirmed widespread increases in the heat content of the world's oceans with extensive warming in the ocean's upper three kilometres (Barnett *et al.*, 2001; Keeling & Garcia, 2002; IPCC, 2013).

Additionally, the uptake of CO_2 by the oceans has resulted in a decline in pH and shift in seawater carbonate chemistry, a process referred to as ocean acidification (OA) (Wittman & Pörtner, 2013). Changes in the planet's climate system are also leading to increased occurrence of marine hypoxic events and changes in salinity. These changes to the physical and chemical properties of seawater may have considerable consequences for marine organisms.

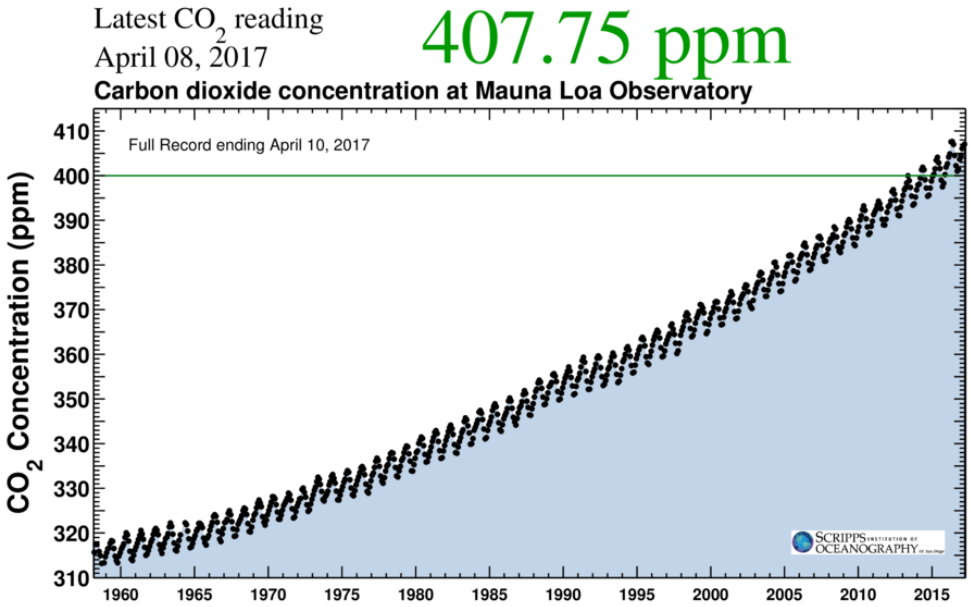


Figure 1.1. Keeling CO_2 curve indicating CO_2 concentration (ppm) over 1958-2017 as measured at the Mauna Loa Observatory in Hawaii, USA (Source: Scripps Institution of Oceanography, UC San Diego)

Climate Change Induced Stressors in the Marine Environments

Ocean Warming

Mean global surface temperatures have risen by 5.4% (0.74°C ±0.18°C) over the past 100 years, with the rate of warming doubling in the past 50 years (Trenberth *et al.*, 2007). Likewise, there is strong evidence that the heat content of the World Ocean has increased since 1955 with surges most obvious in the North Atlantic. Data show that global ocean temperatures have risen by 0.10°C at sea surface to a depth of 700 m since 1963 with the five highest sea surface temperatures occurring since 1995 (Bindoff *et al.*, 2007). The Intergovernmental Panel on Climate Change (IPCC) Fifth Assessment Report (IPCC, 2013) states that ocean warming is greatest near the surface and reports with "virtual certain[ty]" that the upper 75m have warmed by 0.11°C per decade over the period 1971-

2010 (IPCC, 2013). In the United Kingdom (UK) region, the annual sea surface temperature, averaged for the UK coastline, increased by approximately 0.5°C to 1°C between 1870 to 2007 with warming most pronounced in the Southern North Sea, Irish Sea, Minches and Western Scotland (UKMMAS, 2010) (Figure 1.2).

Under the IPCC IS92a CO_2 emission scenario, predictions state that the current average sea surface temperature of 19.7°C will rise to 22.7°C by the end of the century (Houghton *et al.*, 2001; IPCC, 2013). Likewise, under a medium greenhouse gas emissions scenario, UKCP09 (UK Climate Change Projections) projects that UK shelf seas will be 1.5 to 4 °C warmer by the end of the 21st century (UKMMAS, 2010).

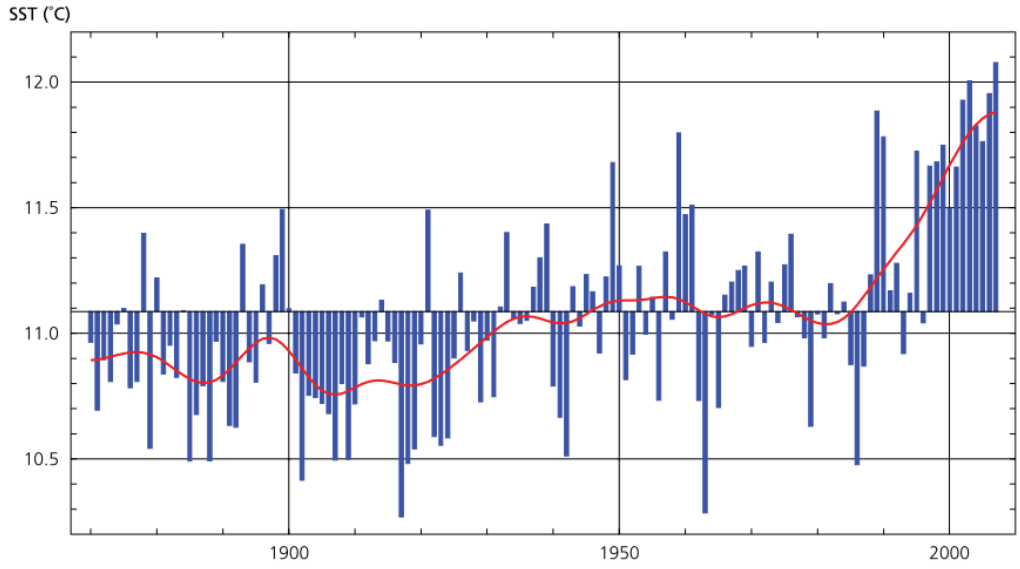


Figure 1.2. Warming trend (mean annual sea surface temperature, SST) for UK shelf seas for the period 1870-2007. Blue bars represent difference from period mean. Red line represents trend line (Source: UKMMAS, 2010).

Hypoxia

Increases in temperature alter the physical, chemical and biological characteristics of seawater. Of particular concern is the reduction of seawater oxygen concentrations that occurs as oxygen becomes less soluble with increasing temperatures (Keeling & Garcia, 2002). There is evidence that oxygen concentrations in the thermoclines of most ocean basins have decreased since the 1970s, likely driven by reduced rates of water renewal (Bindoff *et al.*, 2007). The number of coastal sites where low oxygen conditions (hypoxia) has been reported has increased by 5.54% ± 0.23% yr^{-1} over the past 25 years and future

increases are expected in response to the combined effected of coastal eutrophication and global warming (Vaquer-Sunyer & Duarte, 2008). This is chiefly due to increased water column stratification and warming that inhibits water exchange, enhanced respiration under warming conditions (refer to Chapter 5, pp.189, *Oxygen Consumption*), and increased freshwater input with associated agricultural nutrients (Birchenough *et al.*, 2015). Current climate change projections to the end of this century predict a four to seven percent decline in dissolved oxygen in the oceans (Matear & Hirst, 2003) but even small changes in the oxygen content of marine waters could influence the extent of hypoxic regions in coastal seas, sediments or open ocean areas and lead to such consequences as interruption of the nitrogen cycle and changes in the distribution of many marine organisms (Keeling & Garcia, 2002). Additionally, thermo-stratification due to surface warming may result in reduced nutrient upwelling from deeper waters into surface water thereby reducing photosynthetic production and limiting the downward transport of oxygen into the ocean's interior (Keeling & Garcia, 2002).

Ocean Acidification (OA)

Carbonate buffering is the process by which the oceans have maintained a relatively constant pH for 25 million years, despite the occurrence of high atmospheric $CO_{2(atm)}$ concentrations during that time (Doney, 2006; Widdicombe & Spicer, 2008). However, as availability of carbonate ions depends on geological processes such as erosion which occur over lengthy periods times and current rates of CO_2 diffusion (and subsequent input of H^+ ions) are much higher than usual, there has been a failure of the carbonate buffering system (Widdicombe & Spicer, 2008). The critical result of this upset in equilibrium is an increase in seawater hydrogen ion concentration and thus, a decrease in pH, commonly referred to as ocean acidification (OA).

Data indicate that the pH of seawater has decreased from a pre-industrial era pH of 8.16 to a current pH of 8.05 (Doney, 2006; Guinotte & Fabry, 2008). Since the 1980s, the average pH measurements at the Hawaii Ocean Time-Series, the Bermuda Atlantic Time-Series, and European Station for Time-Series in the Ocean have decreased approximately 0.02 units per decade (Solomon *et al.*, 2007). Furthermore, current models predict that oceanic pH will decrease by further 0.3 - 0.4 pH units (representing a two-fold increase in acidity) by 2100. It has further been suggested that ocean pH in several centuries from

now could be lower than at any time in the past 300 million years (Caldeira & Wickett, 2003).

OA results in a lowered carbonate saturation state (Ω) and consequently, a reduced availability of carbonate for marine calcifying organisms. This is accompanied by an upward shift in the saturation horizon, with the more soluble aragonite saturation horizon shallower than the calcite saturation horizon (Doney, 2006; Fabry *et al.*, 2008). Species at higher latitudes are particularly susceptible to these changes as cold sea temperatures and upwelling of CO_2 rich water make calcification less favourable (Clark *et al.*, 2009). Orr *et al.* (2005) predict that by the end of the century some marine areas of the globe, particularly southern polar regions, with be under-saturated with respect to aragonite.

Salinity Changes

The world's oceans play a key role in the Earth's hydrological cycle and climate (Chahine, 1992). Consequently, any change in a global hydrological process (e.g. precipitation, evaporation, river run-off, sea-ice formation and melt, storage of freshwater in continental glaciers) has a relay effect to oceanic systems (Boyer *et al.*, 2007). Arguably, the most obvious hydrological influence is the effect of any addition and subtraction of freshwater to the ocean salinity (Rahmstorf, 1995). Furthermore, changes in salinity and therefore seawater density have associated effects to vertical stratification, horizontal density gradients, and consequently circulation patterns such as oceanic gyres and residual currents (Malone *et al.*, 2003).

Previously, there has been considerable focus on changes in temperature and pH across marine environments. However, ocean salinity is now being recognised as an important indicator of change in the climate system (Bindoff & Hobbs, 2013). There is growing evidence for climate-induced changes in ocean salinity and an overall acceleration of the global hydrological cycle (Helm *et al.* 2010). Consistently, a pattern of increased salinity in the upper thermocline across all ocean basins has been shown (Solomon *et al.*, 2007) (i.e. freshwater input is decreasing). Specifically, recent analyses of global datasets of *in situ* observations for 1970-2005 showed a global pattern of increased salinities near the upper-ocean salinity-maximum layer (average depth of ~100 m) and decreased salinities near the intermediate salinity minimum (average depth of ~700 m) (Helm *et al.*, 2010).

Evidence suggests that a freshening to a minimum occurred in UK waters during the 1980s-1990s followed by a subsequent increase in salinity (Evans *et al*., 2003; Holliday *et al*., 2008). Irish Sea salinities are especially variable typically falling between 34 and 35 parts per thousand (ppt) in along the western peripheries but falling as low as 31 ppt in areas of large freshwater inputs (UKMMAS, 2010). Overall, trends in the shelf seas of the UK (e.g. Irish Sea) are unclear due to hydrodynamics and a lack of sampling effort at both regional and local levels (UKMMAS, 2010). However, under a medium greenhouse gas emissions scenario, UKCP09 (UK Climate Change Projections) projects that UK shelf seas will be 0.2 salinity units fresher by the end of the 21st century though it should be noted that such projections exist with a degree of uncertainty (UKMMAS, 2010). Additionally, the IPCC states that regions of net precipitation (precipitation greater than evaporation) will likely become fresher, while regions of net evaporation have become more saline (Bindoff & Hobbs, 2013; IPCC, 2013).

Marine Management

Currently, a number of marine management developments are taking place across Europe with the collective aim to safeguard marine resources, ensure future economic sustainability of marine industries, and aid in the conservation of ecologically valuable species and habitats. Predominant marine governance comes from the EU Marine Strategy Framework Directive (MSFD) which calls for "good environmental status" (GES) across marine areas in all member states by 2020. In order to achieve GES, priority habitats (i.e. defined as 'threatened and/or declining species and habitats' under the OSPAR Convention for the Protection of the Marine Environment of the north-east Atlantic 1992) will need to be assessed, maintained and managed for both current conditions as well as future conditions under climate change scenarios (MSFD, 2008). Similarly, the EU Habitats Directive also calls for development of conservation management policy and indicators of condition in Marine Protected Areas (Council Directive 92/43/EEC, 1992). At the same time, planning frameworks at regional and national scales, consisting largely of integrated coastal zone management (ICZM) and marine spatial planning (MSP) are also emerging (Mackenzie *et al.,* 2013). Among various criteria, two vital aspects of management decisions are that they be evidence-based and include adaptations for climate change (MSFD, 2008; Zampoukas *et al.,* 2012). Thus, the generation of scientific knowledge regarding species vulnerability under climate change conditions will be a timely contribution to such efforts.

1.2 Species Overview

Description and Distribution

The marine bivalve *Modiolus modiolus* (Linnaeus, 1758) (horse mussel) is an Arctic-Boreal species with a distribution range that extends from the seas around Scandinavia (including Skagerrak & Kattegat) and Iceland southward to the Bay of Biscay (Rees, 2009). The species also occurs along the Pacific and Atlantic coasts of North America (Brown, 1984). *M. modiolus* is predominantly a sublittoral semi-infaunal species though intertidal populations are occasionally encountered in shallow pools and damp cervices along the lower reaches of the intertidal region (Seed & Brown, 1977).

M. modiolus aggregations, referred to as biogenic reefs, are more limited in their distribution as compared to the species as a whole with current estimates placing the southern limit of such habitats in the southern Irish Sea (Rees, 2009). *M. modiolus* biogenic reef habitats are defined as follows (from Morris, 2015): "*M. modiolus* is the foundation species in biogenic reefs that are characterised by clumped mussels and shell covering more than 30% of the substrate, which may be infaunal or embedded reefs, semi-infaunal (with densities of greater than 5 live individuals per m^2) or form epifaunal mounds (standing clear of the substrate with more than 10 live individuals per clump), all of which support communities with high species richness (or diversity) compared to sediments of the surrounding area". Three key factors have been suggested as of primary importance to defining *M. modiolus* reefs: (i) live adult *M. modiolus* individuals are present; (ii) the biota/communities are distinct from the surrounding habitat and substrates; and (iii) the distinct region containing *M. modiolus* is greater than $25m^2$ in extent (Morris, 2015).

M. modiolus reefs have been found at depths up to 70m, typically in fully saline conditions and tide-swept areas, though a smaller proportion can also be found in sheltered bays, fjords or lochs (Rees, 2009), where temperature and salinity regimes may vary. Likewise, considerable temperature variation exists across the latitudinal range with sea surface and bottom temperatures ranging from 4°C to as high as 19°C over the course of the year (Reid *et al*., 2010). Reefs are found on a range of substrata including cobbles, muddy gravels and sands, where they tend to have a stabilising effect, due to the production of byssal threads by mussels. Such areas can build up as biogenic reefs via accretion of shell and faecal deposits (Lindenbaum *et al.*, 2008; Wildish *et al.*, 1998).

Across the UK, *M. modiolus* reefs are known to occur in Shetland Islands, Orkney Islands, Hebrides and various western and northern coastal areas of the mainland in Scotland; Ards Peninsula and Strangford Lough in Northern Ireland; Point of Ayre, Ramsey Bay and south of Douglas (Little Ness site) in the Isle of Man; and northwest Anglesey and Lleyn Peninsula in Wales (UK BAP, 2008) (Figure 1.3). UK and Isle of Man reefs represent a substantial proportion of all European beds and within the UK, Scotland's coasts support approximately 85% of beds (OSPAR, 2008; Lancaster *et al.*, 2014).

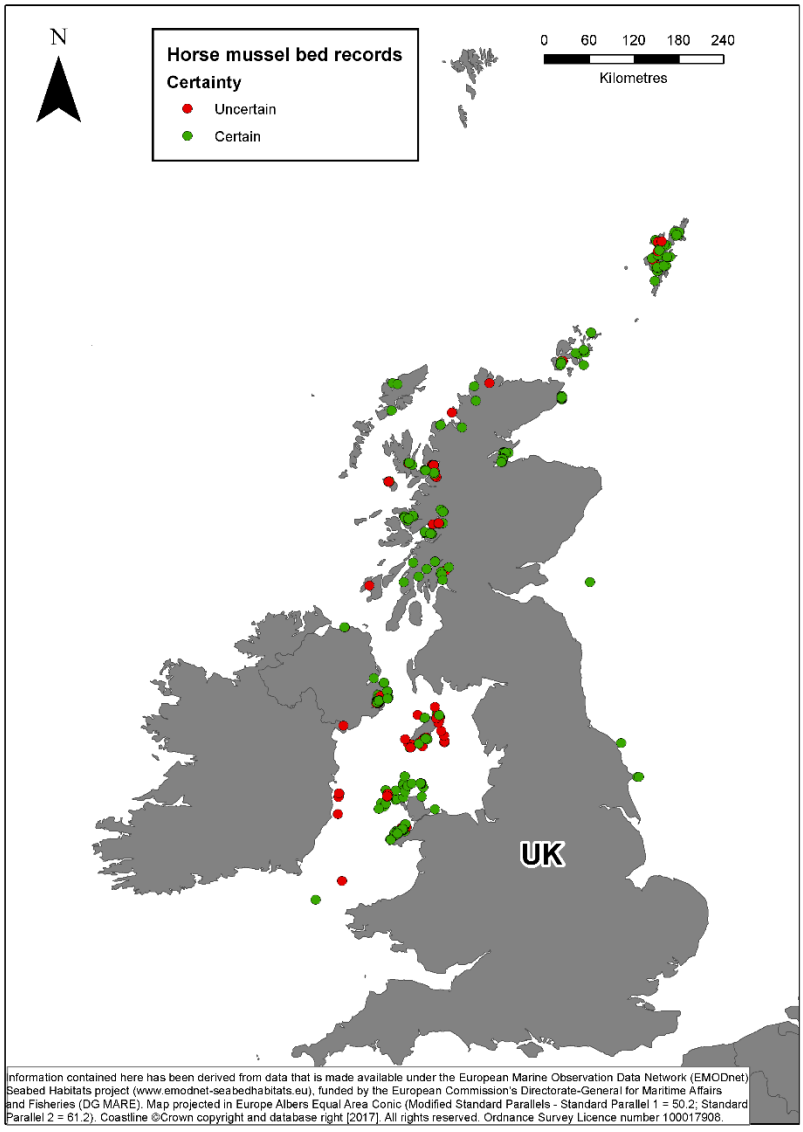


Figure 1.3. Distribution of *Modiolus modiolus* reefs in UK waters (OSPAR records "*Modiolus modiolus* horse mussel beds" from the European Marine Observation Data Network).

Conservation Value

M. modiolus biogenic reefs are characterized by high species diversity (Rees *et al.,* 2008; Sanderson *et al.,* 2008; Ragnarsson & Burgos, 2012). A wide variety of communities are associated with *M. modiolus* reefs including an extensive range of epibiota and infauna such as hydroids, red seaweeds, solitary ascidians and bivalves (Rees, 2009; Ragnarsson & Burgos, 2012). Habitat modification by *M. modiolus* can also have substantial positive effects on the composition and abundance of megafaunal benthic organisms in coastal waters (Ragnursson & Burgos, 2012). In addition to being areas of high biodiversity, *M. modiolus* beds also contribute a number of ecosystem services. These include, but are not limited to, water quality improvements, benthic-pelagic coupling, and seabed stabilisation (Rees, 2009; Kent, 2015). Likewise, the species performs an important role in the energetics of the area it inhabits (Seed & Brown, 1977).

Status

M. modiolus is listed as a threatened and/or endangered species and habitat in all OSPAR regions (OSPAR, 2008) (Figure 1.4). Consequently, *M. modiolus* reefs are considered an OSPAR priority habitat[1] and thus are a conservation priority under the EU Marine Strategy Framework Directive (MSFD; 2008/56/EC), Marine (Scotland) Act and UK Marine and Coastal Access Act 2009. Likewise, *M. modiolus* reef habitats are both a UK Biodiversity Action Plan (now UK Post-2010 Biodiversity Framework) priority habitat (identified as most threatened and requiring conservation action) (UK BAP, 2008) and a priority marine feature under Scottish Natural Heritage (identified as marine nature conservation priorities in Scottish waters) (Hirst *et al*., 2012).

[1]defined as 'threatened and/or declining species and habitats' under the OSPAR Convention for the Protection of the Marine Environment of the north-east Atlantic 1992.

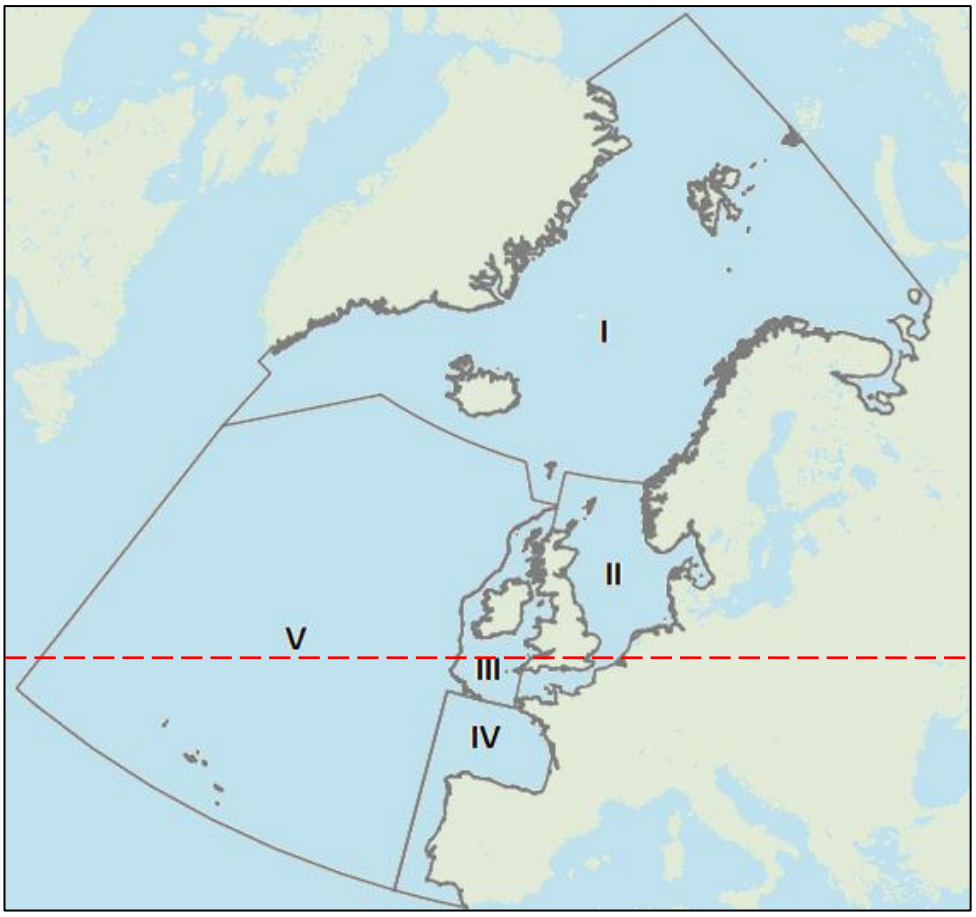


Figure 1.4. *Modiolus modiolus* reefs are listed as threatened or endangered in all OSPAR areas (I-V) and in decline at the southern limit of distribution (approximated by red dashed line) (Source: OSPAR).

Threats to Survival

Historical fishing activity, namely scallop trawling and dredging, has caused widespread and long-term damage to *M. modiolus* reefs across the UK, most notably those situated around the Isle of Man and Northern Ireland (Mair *et al*., 2000; UK BAP, 2008; Rees, 2009; Cook *et al.*, 2013). Such practices directly impact beds by flattening reef mounds, leading to direct fatalities of *M. modiolus* and substantial loss of much associated epifauna (UK BAP, 2008). Cook *et al.* (2013) demonstrated the detrimental impacts of bottom fishing to *M. modiolus* beds where a single pass of fishing gear resulted in significant declines in *M. modiolus* abundance as well as substantial decreases in associated epifaunal diversity and abundance. It should also be noted that even where protection of reefs is in place (e.g. Strangford Lough, Northern Ireland), *M. modiolus* have shown poor recovery possibly due to slow growth of the species and poor recruitment (or self-recruitment) in some populations (Roberts *et al*., 2011; Elsäßer *et al*., 2013).

Less is known about the impacts of climate change to *M. modiolus* reefs and in some cases, it is difficult to disentangle fishing impacts from potential climate impacts (Hiscock *et al*., 2004). Limited prior investigation into the temperature requirements for adult *M. modiolus* suggests an optimal growth temperature of approximately 7-10°C with an upper thermal tolerance limit of about 15-20°C (Halanych *et al.*, 2013). Baseline data for 2009 indicate that mean bottom seawater temperatures across the species' range in the UK fall in the range of 10°C (Gormley *et al*., 2013). However, in the Irish Sea region, mean sea surface temperature can reach values as high as 18°C in summer months (UKMMAS, 2010). Therefore, any decline of populations at the southern end of the range (i.e. southern Irish Sea) does suggest that increasing seawater temperature may be a threat to survival. Recent modelling and GIS environmental envelope analysis of *M. modiolus* biogenic reef habitats also indicates that increased ocean temperature could lead to 100% loss of marine habitats deemed "most suitable" for *M. modiolus* by 2080 (Gormley *et al.*, 2013).

Reduced survival may be a consequence of direct impacts of temperature to physiological mechanisms of the species (e.g. growth rates, oxygen consumption, protein synthesis, immune response) or, be an outcome of the introduction of other stressors including range expansion by competing species, altered or increased parasite/bacterial incidences or changed food availability (Ford, 1996; Cook *et al*., 1998; Heath *et al*., 2012; Callaway *et al.*, 2013; Birchenough *et al.*, 2015). Elevated seawater temperatures may also decrease reproductive output, larval fitness, and survival to maturity of benthic, boreal marine species, especially in those populations at the southern limits of distribution (Dinesen & Morton, 2013; Birchenough *et al*., 2015). In addition to impacts to existing populations, warming may also prevent natural recovery of those populations historically impacted by fishing practices (Hiscock *et al*., 2004).

Sensitivity to Climate Change

Evidence regarding the responses of sub-tidal species to future climate change is currently lacking (Birchenough *et al*., 2013) and empirical evidence regarding climate change sensitivity of *M. modiolus* at population level is virtually non-existent. Limited prior research has demonstrated that ocean warming may have consequences for energetics (e.g. oxygen uptake and metabolism) (Navarro & Thompson, 1996; Anestis *et al.*, 2008; Ezgeta-Balić *et al*., 2011) and aspects of the stress response (Anestis *et al.*, 2008;

Katsikatsou *et al.*, 2012) in *M. modiolus*. Likewise, recent investigation suggests that additional abiotic stressors such as hypoxia, salinity and pH may also impact physiological processes (Bakhmet *et al.*, 2011; Mattoo *et al.*, 2013; Wittman & Pörtner, 2013).

While further information regarding the impacts of climate change to *M. modiolus* is limited or non-existent, a wealth of previous research on other marine bivalve species (e.g. *Mytilus edulis*) can be drawn upon. The impacts of ocean warming, acidification, hypoxia and salinity changes to physiological aspects of bivalve species including growth/calcification, immune status, acid-base balance and metabolism/oxygen uptake (see reviews by Hofmann & Todgman, 2010; Pörtner, 2010; Gazeau *et al.*, 2013; Wittman & Pörtner, 2013), cellular and molecular based responses including changes in cellular energetics, oxidative stress responses and gene expression (Lesser, 2006; Cherkasov *et al*., 2006; Erk *et al.*, 2011; Stillman & Tagmount, 2009) and behavioural and reproductive aspects (Harley *et al.*, 2006) have all been demonstrated. However, as *M. modiolus* is both ecothermic (i.e. a thermo-conformer) and sub-tidal (i.e. inhabiting a buffered environment), the species may be less physiologically capable of adjusting internal mechanisms to cope with these sudden changes than some of its counterparts (Whiteley & Mackenzie, 2016). As comparison, the intertidal species *Mytilus edulis* regularly experiences thermal and dessication stress (with tidal cycles) and consequently has improved capacity to tolerate stressors (Whiteley & Mackenzie, 2016). For example, *M. edulis* has an upper thermal tolerance that is approximately 4°C higher than *M. modiolus* (Read & Cumming, 1967).

Global warming of the oceans will not occur in isolation and it is becoming increasingly evident that the emergence and increasing intensity of various additional climate-induced stressors in marine environments including changes in pH (i.e. ocean acidification), oxygen content (i.e. hypoxia) and salinity will interact with the effects of warming to further influence physiological responses and their effect on thermal tolerances (Hofmann & Todgman, 2010; Pörtner, 2010; Gazeau *et al.*, 2013;). For examples, *M. modiolus* reefs are predominantly located in coastal areas and therefore will also be increasingly exposed to coinciding warming and hypoxia, particularly those populations situated in sheltered (i.e. reduced wave energy and water exchange) areas such as fjord or sea lochs (Reid *et al.*, 2010). Therefore, the impacts of multi-stressor conditions are an important

consideration. Any such effects could yield serious consequences for the biogeography of species and thus, will be important considerations for management of the species (Heath *et al.*, 2012).

Emerging research highlights the importance of considering climate change impacts across life-stages. As larval health and development is highly contingent on parental health and physiology, indirect impacts to a parent may present in the development and functioning of the larval stages (Clark *et al.,* 2009). Consequently, where climate change impacts at the adult life stage may be absent or minimal, considerable effects to larval stages and/or future adult generations may occur (Hofmann & Todgham, 2010; Talmage & Gobler, 2010). Unfortunately, there remains a critical absence of data on the impacts to larvae from adults reared in elevated CO_2 conditions. This is particularly noted for *M. modiolus*, for which current understanding of larval behaviour and development is poorly understood or varies considerably between locations/populations (Holt *et al.,* 1998; Roberts *et al.,* 2011), and thus making it difficult to investigate impacts to larval life stages or across generations. Additionally, spawning and larval rearing techniques for the species remain poorly understood and developed (though Roberts *et al*., 2011 provide important foundational information). Consequently, the current work limits investigation to adult life stage but emphasises an important direction for future *M. modiolus* research.

1.3 Defining Vulnerability

Critical to understanding the impacts of climate change to any given marine species is the obtaining of an understanding of that species' vulnerability to a changing environment. Zacharias & Gregr (2005) define vulnerability as "the *probability that a feature will be exposed to a stress to which it is sensitive"* where sensitivity is *"the degree to which marine features respond to stresses, which are deviations of environmental conditions beyond the expected range."* Vulnerability can be further described as a function of the level of stressor imposed on a given populations/species (i.e. exposure), the capability of a population to adapt to the stressor (i.e. adaptive capacity), and the ability of that population/species to respond to the stressor (i.e. acclimatory ability), represented as: Vulnerability = *f*(exposure, adaptive capacity, acclimatory ability). This vulnerability function provides a framework for the current investigation of the impacts of climate change to *M. modiolus* (Figure 1.5).

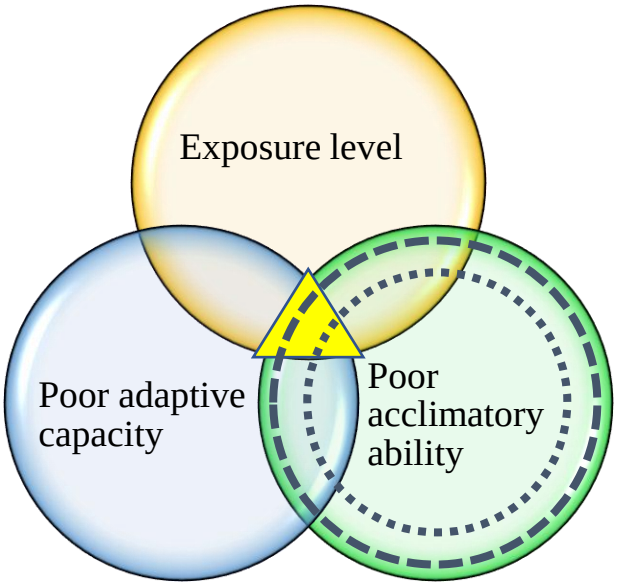


Figure 1.5. Vulnerabilty framework illustrating the relationship of adaptive capacity, acclimatory ability and level of exposure in determining climate change vulnerability (yellow triangle). The dashed inset circles demonstrate how varying strengths of a factor can impact level of vulnerability. Schematic adapted from Maxwell *et al*. (2015).

Adaptive Capacity

In marine environments, various physical factors including temperature, salinity, current speed, and turbidity, vary enormously across species' distributions. Consequently, adaptive variation tends to emerge between populations situated across environmental gradients, or between populations that have limited connectivity (Whitehead *et al*., 2011; Calosi *et al*., 2017). In this way, climate change may potentially restructure ecosystems as species' distributions are influenced by population-based tolerances and adaptation to stressors. This is particularly noted for temperature, which is a well acknowledged physical driver for setting species' abundance and distribution limits, with changes in such factors frequently observed at the edge of species' ranges where organisms are more likely to experience high levels of physiological stress (Hofmann & Todgman, 2010; Birchenough *et al.*, 2015; Calosi *et al*., 2017).

If environmental conditions exceed the physiological capabilities of some individuals, then natural selection may favour genotypes of alternative individuals (i.e. the "fittest"). Further, if conditions exceed the ability of all individuals, the population will likely not survive or will be pushed to adjust to more favourable conditions (i.e. range shift), if such

a move is possible (Helmuth *et al.*, 2005). Give that there are various environmental factors acting as selective pressures on a given population, spatial differences in thermally (or other) tolerant genotypes may be complex and prove a challenge to determining a given species climate envelope under future climate conditions (Kuo & Sanford, 2009; Calosi *et al*., 2016).

Acclimatory Ability

Acclimatisation (or *acclimation* under laboratory conditions) refers to the plasticity of organisms to react to environmental conditions without genetic changes (Rost *et al*., 2008). It may be any physiological or biochemical change that occurs within the lifespan of an individual as a result of exposure to new conditions in the animal's environment (Willmer *et al*., 2009). Over periods of weeks or month, acclimatisation may shift physiological mechanisms (e.g. acid-base regulation, respiration rates and calcification rates) to compensate for environmental stressors (Pörtner, 2008).

The extent to which changes in the expressed phenotype of a genotype occur as a function of the environment is referred to as physiological plasticity (Schiener, 1993). Levels of physiological plasticity can aid in determining the range of environmental conditions to which an organism/population may acclimatise (Helmuth *et al.*, 2005; Whitehead, 2012) and the inherent sensitivity of a population under such conditions. Where phenotypic plasticity does occur, a population may also be provided additional time to rapidly adapt (Calosi *et al*., 2016). Furthermore, physiological acclimatisation of populations to a particular set of environmental conditions is correlated with increasing genetic differentiation between those populations (Pörtner, 2002). Such adaptive divergence can occur over a range of spatial scales (from meters to hundreds of kilometers) (Sanford & Kelly, 2000).

Marine species may possess the ability to acclimatise to changing environmental conditions and hence have the potential to increase tolerances to environmental stressors (Somero, 2010; Whitehead, 2012; Calosi *et al*., 2013). For example, Calosi *et al*. (2013) demonstrated physiological plasticity in the metabolic response of a marine polychaete, *Amphiglena mediterranea*, under elevated pCO_2 conditions. Still, it remains relatively unclear whether or not *M. modiolus* can adjust to changing conditions. As a bivalve, the species possesses poor ability to regulate physiological temperature, osmoregularity, and

pH, and thus has a physiology that is directly impacted by changes in seawater temperature, salinity or pH. Furthermore, even highly heat-tolerant ectotherms may be threatened by further increases in temperature, due to proximity of these species' thermal optima and tolerance limits to current maximal ambient temperatures and limited capacities for acclimatization to higher temperatures (Somero *et al.*, 2011). Given that seawater temperatures in some areas of the *M. modiolus* species' range represent values near the upper tolerance limits for the species (e.g. Lleyn Peninsula, Wales), it is critical that investigation into the acclimatisation limits of the species under future climate change conditions be conducted.

Stress Response

A foundational understanding of physiological mechanisms and how any given environmental stressor will alter physiological tolerances and performance of a species or population (i.e. the "stress response") is imperative for determining sensitivity. Knowing where an animal exists today within its "tolerance thresholds" and to what degree predicted climate change will shift this position may allow us to assess the degree of vulnerability for a species (Hofmann & Todgman, 2010). The stress response in marine invertebrates is comprised of numerous biological aspects. These include physiological (Pörtner *et al*., 2004; Somero, 2010; Kelly & Hofmann, 2012), immunological (Malagoli *et al.*, 2007; Ellis *et al.*, 2011), energetics-based (Cherkasov *et al*., 2006, Ezgeta-Balić *et al.*, 2011, Sokolova *et al.*, 2012) and behavioural components (Monari *et al*., 2007). Likewise, responses may manifest across varying levels of cellular organisation and life stages and thereby go undetected should the given level or stage fail to be considered (Pörtner, 2012). In this thesis, attention is paid to two specific components of the stress response: (i) oxidative stress response; and (ii) changes in energy uptake, consumption and budgets across varying levels of cellular organisation.

Oxidative Stress Response

The oxidative stress response refers to the production and accumulation of reduced oxygen intermediates in response to exposure to environmental stressors including thermal stress, ultraviolet radiation or pollution (Ahmad, 1995; Lesser, 2006) and is a critical part of the overall stress response in a living organism. Thermal (or other climate induced) stressors at the molecular level of organismal physiology may provide the underpinning for impacts occurring at higher levels of organisation including organelle,

cell, tissue and systemic stages (Pörtner, 2002). Consequently, such information is an important contribution to the developing knowledge base of molecular to whole-organism trade-offs and restrictions for acclimatization and evolutionary adaptation under conditions of environmental stress (Pörtner *et al.*, 2010; Mattoo *et al.*, 2013).

Impacts on Energetics

Under increased thermal stress (e.g. at the edge of a biogeographic range), strain is placed on internal physiological systems associated with recovery and maintenance of homeostasis. The related costs of energy acquisition, conversion and conservation impact energy budgets, with negative consequences for growth and reproduction (Sokolova *et al*., 2012). There are numerous previous studies that demonstrate temperature-induced changes to various aspects of energetics including growth, metabolism, reproductive output, and condition index in ectotherm species (Pörtner, 2002; Lesser & Kruse, 2004; Pörtner, 2012; Sokolova, 2013; Hofmann & Todgham, 2010). Therefore, consideration of changes to energetics under climate change stressors is important to determining the overall vulnerability of *M. modiolus*.

1.4 Project Aims

There is a clear need for evidence-based information regarding the current and future status of *M. modiolus* biogenic reef habitats under climate change conditions in order to properly inform marine management of the species, aid marine planning developments and meet governance requirements. While *M. modiolus* faces threats from a number of climate change stressors (as previously outlined), the main focus of this investigation will be the effects of warming. Existing data and prior research show a high certainty of increased seawater temperature across *M. modiolus'* distribution and there is also high likelihood that *M. modiolus* (as a thermo-conforming and non-motile species) will be threatened by such increases. Additionally, as temperature maxima (and therefore risk) under climate change conditions will vary over latitude, focus on warming impacts will provide a unique opportunity to investigate how vulnerability may vary across populations. Finally, as temperature is a key driver of species distribution, consideration of temperature impacts will be a valuable contribution to management efforts, particularly regarding marine spatial planning (e.g. designation of Marine Protected Areas). In addition, hypoxia will be examined as a co-stressor, principally as increased occurrence of low oxygen conditions is strongly linked to temperature change (i.e. due to the inverse relationship between oxygen content and temperature). Moreover, instances of hypoxia are associated with loch environments where a high number of *M. modiolus* reefs are located (e.g. Scottish west coast).

Further understanding of each of the factors that influence climate change vulnerability (i.e. exposure, adaptive capacity and acclimatory ability) at both species and population levels and across varying scales of organisation (cellular-whole organism) will generate valuable information and tools that may inform and aid management measures. To achieve this, the main aims of this thesis are as follows:

(1) To examine the role of the environment in shaping *M. modiolus* population-based vulnerability to climate change stressors (warming and hypoxia) via collation of past and future temperature and dissolved oxygen conditions across the species' range and at select reef sites; **(EXPOSURE)**

(2) To examine genetic structure and connectivity of *M. modiolus* populations across the species' UK distribution; **(ADAPTIVE CAPACITY)**

(3) To characterise the acclimatory ability of *M. modiolus* via investigation of various aspects of the stress response (e.g. oxidative stress response including oxidative damages and antioxidant production, energetics, physiological limits) of *M. modiolus* at species and population levels under simulated climate change conditions in the laboratory; **(ACCLIMATORY ABILITY)**

(4) To consider outcomes of (1) – (3) to inform management for *M. modiolus* reef habitats.

1.5 Thesis Structure

The following chapters (2-6) are laid out to meet the above objectives. A brief overview of each chapter and identification of the objective(s) met by that chapter are provided, as follows:

Chapter 2 (*Objective 1*) examines the physical (i.e. abiotic) characteristics of *M. modiolus* reef sites from across the UK range by delving into historical records and making use of contemporary measurements of sea water temperature and dissolved oxygen levels. Broad scale and, where available, site specific monitoring data were collated and are presented for key reef sites across the UK range of *M. modiolus* reef habitats. Additionally, climate change conditions according to current projections (e.g. IPCC) were determined for a number of sites to indicate potential levels of future climate stress. A summary of current and future conditions is presented as contribution towards an understanding of the "environmental basis" for vulnerability.

Chapter 3 (*Objectives 2 and 4*) examines the adaptive capacity of *M. modiolus* as per genetic connectivity and diversity analyses. Laboratory-based microsatellite analyses were carried out for *M. modiolus* populations from across the UK distribution to assess population diversity and connectivity. Preliminary genetic connectivity and diversity investigation focused on key UK populations and included application of connectivity analyses for determination of transplant suitability in Northern Ireland populations. This work was followed up with more comprehensive (i.e. increased numbers of markers and sites) connectivity and diversity analyses to meet management needs via involvement with (1) Scottish Natural Heritage, to investigate the role of Scottish MPAs in maintaining

the larger network; and (2) Isle of Man Government, to investigate of the role of the Isle of Man Little Ness MPA site in the IOM and wider UK networks.

Chapter 4 (*Objectives 3 and 4*) provides an overview of the impacts of climate change to the stress response in *M. modiolus*, focusing on the oxidative stress response and thermal limits. Acclimation abilities were determined for *M. modiolus* populations from across the UK via a series of short term (week duration) and longer term (month duration) laboratory-based experiments. Biomarkers included antioxidant production, lipid peroxidation, protein carbonylation and DNA damage. Thermal limits were compared across three key populations (representing the southern, mid and northern ranges of the species) and the impact of hypoxia to thermal limits was examined in a single population.

Chapter 5 (*Objective 3*) continues examination of the impacts of climate change to the stress response in *M. modiolus*, but shifts focus to energetics. The impacts of warming and/or hypoxia to varying aspects of energetics (ranging from cellular to whole body) were investigated. A number of laboratory-based physiological experiments were carried out to assess changes in cellular energy allocation, oxygen consumption and condition index under controlled changes in temperature, and/or hypoxia. Additionally, the role of food availability (e.g. low vs. high ration under varying temperatures) to energetics was considered.

Chapter 6 (*Objective 4*) summarises key findings and discusses how findings may be applied to guidance for effective management of *M. modiolus* under climate change conditions. A prototype climate change vulnerability index is provided that may be carried forward by marine managers. Additionally, consideration of *M. modiolus* reef habitats within a template regional management plan is provided as part of an internship project that was carried out under the supervision of Scottish Natural Heritage.

2. **Exposure to Climate Change Stressors:** Past and future temperature and dissolved oxygen conditions across UK *M. modiolus* reefs

2.1 Abstract

The sub-tidal environments inhabited by *M. modiolus* are characterised as stable buffered environments. Such constancy in conditions has a potential adaptive consequence of reduced plasticity in the eco-physiological responses of *M. modiolus* to environmental changes. Consequently, the species may be particularly vulnerable to changes in physical factors such as warming and decreased oxygen content (e.g. hypoxic events) as brought on by climate change. However, on closer inspection, emerging research shows that *M. modiolus* reef populations inhabit a range of habitats that vary in depth, latitude and exposure, amongst other factors, and therefore experience a range of temperatures and dissolved oxygen levels. Populations that are historically accustomed to more stressful conditions and/or increased variability of abiotic conditions may have adaptations or increased acclimatory ability (i.e. plasticity) to cope with changing conditions. Additionally, the intensity of climate change varies across sites and accordingly location will dictate the level of threat posed to populations. In this chapter, data regarding local and regional historical environmental conditions and climate change projection data were collated in order to examine the environmental basis for *M. modiolus'* vulnerability to climate change. Key focus is paid to differences/similarities in temperature and, to a lesser degree, oxygen content between reef sites. Results are used to highlight those populations that may have increased resilience and those that may be at increased risk of climate change, and concludes with a summary table and visual for assessing the general risk of climate change *to M. modiolus* reef populations according to physical conditions.

2.2 Aim and Objectives

Aim:

- Determine current and future temperature and dissolved oxygen conditions at *M. modiolus* reef sites across the UK and Isle of Man range of the species so as to examine potential influences on adaptive capacity or acclimatory ability and to highlight sites with greater vulnerability under climate change scenarios.

Key Objectives:

- **Illustrate distribution** of *M. modiolus* species and biogenic reef habitats across the UK and Isle of Man to demonstrate variation in location (and therefore site conditions);

- **Collate and summarise physical data** (e.g. seawater temperature and dissolved oxygen content), where available, for *M. modiolus* reef sites across the UK range of the species using both broad-scale regional and site-specific local data to examine differences in conditions and variability across sites;

- Use available projection data to **approximate site conditions under future climate change** conditions;

- **Highlight *M. modiolus* sites that may be at greater risk of climate change** threat according to site conditions or may have increased resilience due to historical exposure to extreme/variable conditions.

2.3 Introduction

Although *M. modiolus* has a widespread pan-boreal distribution, segregated reef populations occur sporadically and are increasingly limited towards the geographic limits of the species (Wales, UK) (OSPAR, 2009). Still, reef sites in the UK and Isle of Man cover a considerable latitudinal range extending as far south as the Lleyn Peninsula (Wales) and as far north as Shetland Islands (Scotland). The sub-tidal environments that *M. modiolus* typically inhabits are characterised as 'stable' with regard to abiotic conditions, particularly when compared to the highly variable inter-tidal zone inhabited by other mussel species (e.g. *Mytilus edulis*). This relative constancy of physical conditions is a chief factor accounting for the increased longevity of *M. modiolus* but also has a potential adaptive consequence of reduced plasticity in eco-physiological responses to environmental changes (Somero, 2010). One would therefore expect any environmental change (e.g. climate change) to have a substantial impact on *M. modiolus* (Ezgeta-Balić *et al.,* 2011).

However, when the distribution range of a species is spread across a diverse landscape, as is the case with *M. modiolus*, spatial variation inevitably leads to spatially distinct populations inhabiting areas with a particular (and often unique) set of parameters (Luttikhuizen *et al.*, 2003; Osovitz & Hofmann, 2007). Environmental conditions associated with *M. modiolus* reefs are often localised and the resulting variability between sites has posed a challenge to defining the ecological limits of the species (OSPAR, 2009). For example, while *M. modiolus* is most commonly found in sub-tidal areas ranging from the lower shore to depths of up to 70m, populations also occur at great depths (in some instances up to several hundred meters) or, in contrast, in intertidal areas, though the latter is rare (OSPAR, 2009). Intertidal and shallow sub-tidal populations experience a substantial range of temperatures across the year (i.e. seasonally, diurnally) whereas deeper sub-tidal population are markedly buffered from large variations in temperature (Whiteley & Mackenzie, 2016). Similarly, while *M. modiolus* reefs exist in current-swept fully-saline and highly oxygenated marine environments, populations also exist in sheltered bays, fjords or lochs where factors such as salinity and temperature are regularly altered by the influx of terrestrial run-off (OSPAR, 2009) and where extended water residence time, reduced water exchange/mixing and increased incidence of eutrophication can lead to lowered levels of dissolved oxygen (e.g. hypoxic events). Hypoxic water masses are defined as having <2mg oxygen L^{-1} (approximately 30% saturation) (Rabalais

et al., 2010). The species also experiences a wide range of temperature regimes over its broad distribution range with seasonal temperatures ranging from -1.8°C (0–1 m depth) and 0°C (up to 30 m depth) to 18–20°C (0-10 m depth) (Lesser & Kruse, 2004). Therefore, while the general description of a *M. modiolus* reef habitat suggests a stable, unchanging environment, there is considerable variation in local conditions when specific populations/sites are considered.

The localised variation in environmental conditions that exists across the UK distribution of *M. modiolus* reef sites suggests that populations experience distinctly different levels of exposure to environmental stressors. While it is generally assumed that populations at the edge of species' ranges are likely to be the most physiologically stressed and thus, impacts will be initially observed in such popualtions, it has also been suggested that local and regional heterogeneity across poulations may lead to observed changes in populations that are situated away from range limits (Birchenough *et al.*, 2015). For example, *M. modiolus* populations at the southern limit of the range will tend to experience warmer temperatures (that peak during the summer season) than other more northern populations. However, as temperature also varies with such factors as water depth and proximity to coastal processes (e.g. excessive freshwater run-off into semi-enclosed basins) populations situated within shallow coastal and enclosed lochs, for example, may experience higher/lower temperatures than more southern/northern populations (Rees, 2009). Similarly, coastal and shallow populations may be more accustomed to (and therefore have adaptation/acclimation to) increased temperature variability as per tidal cycles, input of freshwater from surrounding land masses or proximity to anthropogenic thermal influences (e.g. power plants) than offshore counterparts situated in deeper and therefore more buffered waters.

Climate change will concurrently alter a number of abiotic factors in marine environments and the threat posed by these changing conditions will vary according to location. For example, *M. modiolus* situated at the southern limit of reef habitats (Lleyn Peninsula, Wales), and therefore already existing near the species thermal limit, may be more threatened by temperature increases than more northern populations. Similarly, populations based in coastal lochs where summer stratification regularly occurs and where reefs can exist in proximity to fish farms, may be at increased risk of hypoxic events due to increased occurrence of eutrophication and coinciding warming (Rees,

2009; Rabalais *et al*., 2010). Additionally, populations that have historically been exposed to more extreme/variable conditions may have developed an increased resilience to changing conditions. Such variability in specific environmental conditions associated with a given region will influence the adaptive capacity and/or physiological plasticity of any local populations and will contribute to the range of environmental conditions to which it can tolerate (Whitehead, 2012).

A broadcast species-level approach to determining "level of climate change threat" according to generalised environmental conditions is not necessarily appropriate nor useful for management purposes. Instead, when designing research questions, it is important to consider how climate change will impact conditions on a local or regional basis, in order to accurately advise on the threat posed to specific populations. The aim of this research was therefore to examine the distribution of reef sites across the UK and provide an indication of past and future site conditions (with specific focus on temperature and dissolved oxygen content) on a site/regional basis. Historical and projected temperature and dissolved oxygen data were used to accurately inform on the level of threat posed by climate change, and further, to identify populations where historical conditions may contribute to improved resilience to climate change. Understanding such factors that govern patterns of distribution of sessile benthic species like *M. modiolus* on a site/regional basis will contribute to both marine spatial planning efforts and climate change monitoring efforts (Rouse *et al*., 2014).

2.4 Methodology

Distribution

M. modiolus species presence data were searched for in the National Biodiversity Network (NBN) Gateway website (NBN, 2016). Using the grid map tool, search criteria were set for "*Modiolus modiolus*" and returned 4078 records. A grid map was then created for the UK and Ireland (and included the Isle of Man) for the years 1600-2016 (default) with resolution set to 2km grid squares. Records from Scottish Natural Heritage (n=581), Joint Nature Conservation Committee (n=843), Natural Resources Wales (n=225) and Marine Biological Association (n=165) were displayed. Additionally, a *M. modiolus* reef distribution map was created by importing latitude and longitude coordinates of reef locations into ArcMap. Latitude and longitudes were obtained for a

list of well-known reef sites as collated from reports (Mair *et al*., 2000; 2010; Hirst *et al*., 2012) and personal communications (pers. comm. Heriot-Watt Scientific Dive Team).

Historical Conditions

Temperature

Regional temperature data were downloaded from the National Oceanic and Atmospheric Administration (NOAA), National Centre for Environmental Information (NCEI) website (NOAA, 2016). Statistical means for seasonal (winter, spring, summer and autumn) seawater temperature at a resolution of 1/10° were downloaded from the NCEI Greenland, Iceland and Norwegian Seas (GINS) regional climatology dataset which includes coverage of the UK, Isle of Man and Ireland. The GINS dataset is a regional climatology based on the World Ocean Database archive of temperature observations with temporal coverage of more than one hundred years (NOAA, 2016).

Additionally, temperature data were obtained from the Marine Scotland *Annual Cycles of Physical, Chemical and Biological Parameters in Scottish Waters* dataset (Slesser & Turrell, 2013). The data represent annual observations made by the Marine Laboratory, Aberdeen for the period 1960-2010 and are available for 26 regional areas of Scottish coastal and oceanic waters. Annual temperature data were downloaded from the Marine Scotland website in .csv table format for seven regions that either contain or in close vicinity to well- documented *M. modiolus* reefs sites (Table 2.1). The accuracy of this dataset is +/- 0.02ºC.

HOBO temperature loggers (ONSET, Massachusetts, USA) were also placed by Heriot-Watt Scientific Dive Team onto several reefs sites including Karlsruhe (Orkney Islands), Loch Creran (west coast of Scotland) and North Lleyn (Wales) and left to record over periods of one year (2011-2012), one year (2013-2014) and four years (2003-2008), respectively. A temperature recording was made once per hour.

Please note: It is acknowledged that other sources of temperature data (e.g. CEFAS Coastal Temperature Network) for the geographic area do exist and could be included in future work.

Dissolved Oxygen

Dissolved oxygen data were obtained from the Marine Scotland *Annual Cycles of Physical, Chemical and Biological Parameters in Scottish Waters* dataset (Slesser &Turrell, 2013), as described above. However, it should be noted that accuracy of this dataset is +/- 10%.

Table 2.1. Scottish regions (area, region name) containing or in proximity of *M. modiolus* reef sites. Temperature data were downloaded for listed regions (Source: Marine Scotland)

Area	**Region Name**	**Reef sites***
Coastal Waters - Shetland Islands	Sullom Voe	Calback Ness
	Yell and Fetlar	Hascosay Sound Uyea Sound
Coastal Waters - West Coast	Clyde Sea	Loch Long
Shelf Waters - West Coast	Malin Shelf – Inner	Oban Port Appin Loch Creran
	North Minch	Loch Duich Loch Alsh
Shelf Waters - North Coast	North Coast-North	Karlsruhe
	North Coast-South	Noss Head
Coastal Waters – East Coast	Inner Moray Firth	Dornoch Firth

* *M. modiolus* reefs either contained within region's boundaries or in close vicinity.

Climate Change Conditions

Temperature

Temperature projection data were downloaded via the user interface of the UKCP09[2] (UK Climate Projection) website (Met Office & Environment Agency, 2016). The *Past and*

[2] UKCP09 was created in 2009 and is funded by a number of UK agencies. Data are based on sophisticated scientific methods provided by the Met Office, with input from

future multi-level ocean model simulations for UK Waters data source was chosen to obtain seasonal (Winter, Spring, Summer, Autumn) temporal averages for future absolute climate values. Near bed (approx. 25m) maximum daily mean sea water potential temperature and mean sea water potential temperature data under a medium emissions scenario for 2070-2099 were obtained. All datasets were imported into ArcMap version 10.0 (ESRI, Redlands, California) as shapefiles and added to a UK basemap overlaid with *M. modiolus* reef sites (latitude, longitude point data). The ArcMap select tool was used to isolate temperature values greater than or equal to 15°C in order to illustrate areas where warming was representative of *M. modiolus*' upper thermal limits (i.e. 15-20°C) (Halanych *et al.*, 2013). Additionally, a temperature increase of 4°C as per the IPCC IS92a CO_2 emission scenario was applied to annual temperature data obtained via temperature loggers (see above for details) for North Lleyn, Loch Creran and Karlsruhe beds to provide a general indication of potential warming to be expected at specific sites.

Dissolved Oxygen

To date, no dissolved oxygen projection data exist specifically for the UK marine region but an indication of general patterns is provided by a range of models which project a decline in total ocean inventory of dissolved oxygen by 2-4% (Matear & Hirst, 2003; Bop *et al.*, 2013; Cocco *et al.*, 2013). However, given the limited dissolved oxygen data available for sites/regions, this percentage decline was not applied to data. Instead, populations located in areas associated with thermal stratification in summer months and with historic risk of hypoxia events are discussed.

2.5 Results

Distribution

Figure 2.1 shows species recorded presence for *M. modiolus* in the UK, Ireland and the Isle of Man as compiled by the National Biodiversity Network (NBN, 2016). *M. modiolus* species are present across much of the UK coastline. However, greater densities exist in Scotland off the mainland west coast, Orkney Islands and Shetland Islands. *M. modiolus* reef sites of the UK and Isle of Man are listed and illustrated in Table 2.2 and Figure 2.2.

over 30 contributing organisations. UKCP09 provides future climate projections for land and marine regions as well as observed (past) climate data for the UK.

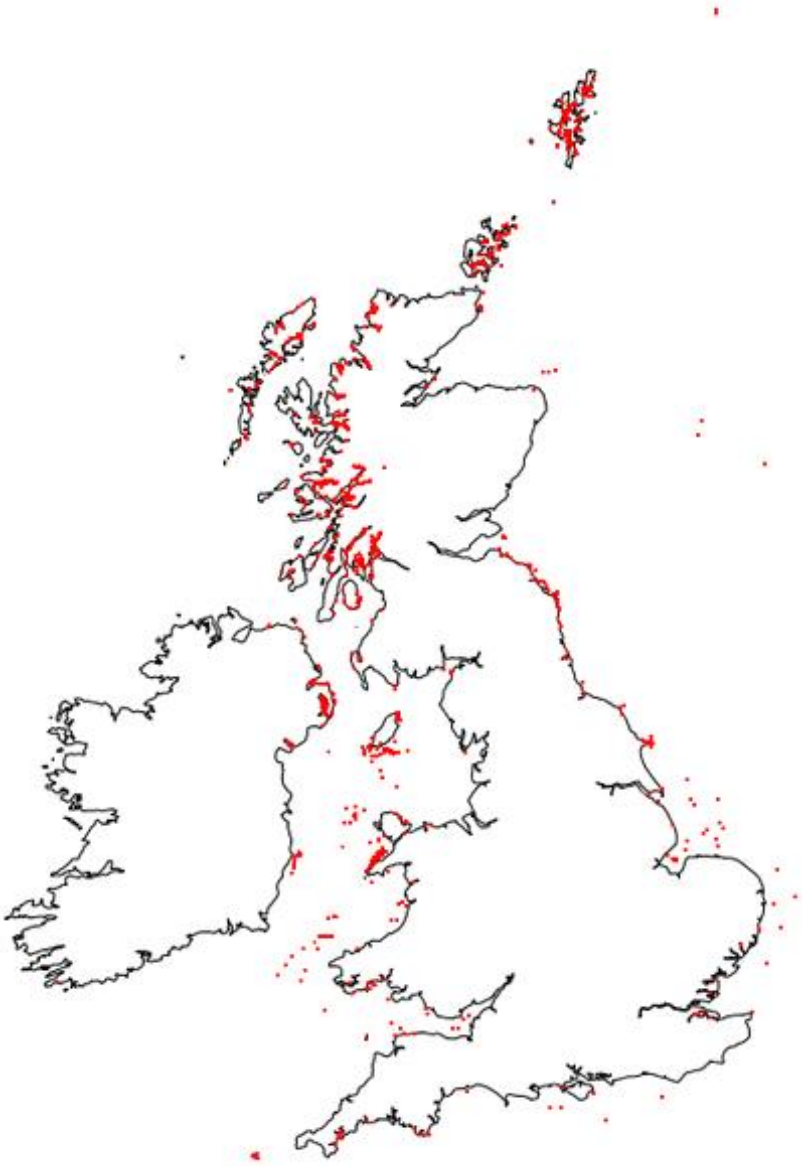


Figure 2.1. *M. modiolus* species presence (2km grid) across the United Kingdom, Isle of Man and Ireland, as obtained from the National Biodiversity Network Gateway. Disclaimer: The information used here was sourced through the NBN Gateway website and included the following resources: Joint Nature Council Committee. "Marine Nature Conservation Review (MNCR) and associated benthic marine data held and managed by JNCC (843)". Updated 22/04/2005; Scottish Natural Heritage. "Species data for Scottish waters held and managed by Scottish Natural Heritage, derived from benthic surveys 1993 to 2014." Updated 7/08/2015; Marine Biological Association. "DASHH Data Archive Centre – Statutory Surveys." Updated 02/201; Natural Resources Wales. "Marine data from Natural Resources Wales (NRW) Technical Support (Research & Monitoring) Contracts, Wales." Updated 13/11/2015. <http://data.nbn.org.uk/> (Accessed 4 Apr. 2016). The data providers and NBN Trust bear no responsibility for the further analysis or interpretation of this material, data and/or information. Crown copyright and database rights 2011 Ordnance Survey.

Table 2.2. *M. modiolus* reef populations of the UK and Isle of Man. Populations are listed according to geographical region and in order of latitude (north-south). Note: This is not an exhaustive list but provides those reef sites that are well-documented and illustrates the geographical range of *M. modiolus* reef habitats across the UK and Isle of Man.

Geographical Region	Site Name	Latitude	Longitude	Depth (m)*
Scotland: Shetland Islands	Uyea Sound	60.67	-0.94	27[d]
	Hascosay Sound	60.62	-1.01	8-25[d]
	Calback Ness	60.48	-1.28	27[a]
	Busta Voe	60.38	-1.36	14[b]
Scotland: Orkney Islands	Karlsruhe	58.89	-3.19	24[c]
	North Cava	58.89	-3.18	24[b]
	Southeast Risa	58.86	-3.19	nd
Scotland: North-East Coast	Noss Head	58.47	-3.02	35-45[c]
	Dornoch Firth	57.86	-4.06	20[c]
Scotland: West Coast	Loch Alsh	57.26	-5.62	20[b]
	Loch Duich	57.24	-5.47	nd
	Port Appin (Loch Linnhe)	56.55	-5.42	20[c]
	Loch Creran	56.55	-5.27	24[c]
	Oban (Loch Leven)	56.41	-5.49	13[c]
	Loch Long	56.03	-4.89	nd
Northern Ireland	Ards Peninsula	54.58	-5.46	nd
	Strangford Lough	54.45	-5.59	25[c]
Isle of Man	Point of Ayre	54.44	-4.31	33[c]
	Ramsey Bay	54.40	-4.34	nd
	Little Ness	54.13	-4.48	28[c]
Wales	North Lleyn	52.94	-4.65	20-30[c]

*depth approximated from [a] Mair *et al.* (2010); [b] Mair *et al*. (2000); [c] Heriot-Watt Scientific Dive Team (pers. comm.); [d] Hirst *et al*. (2013); nd=no data.

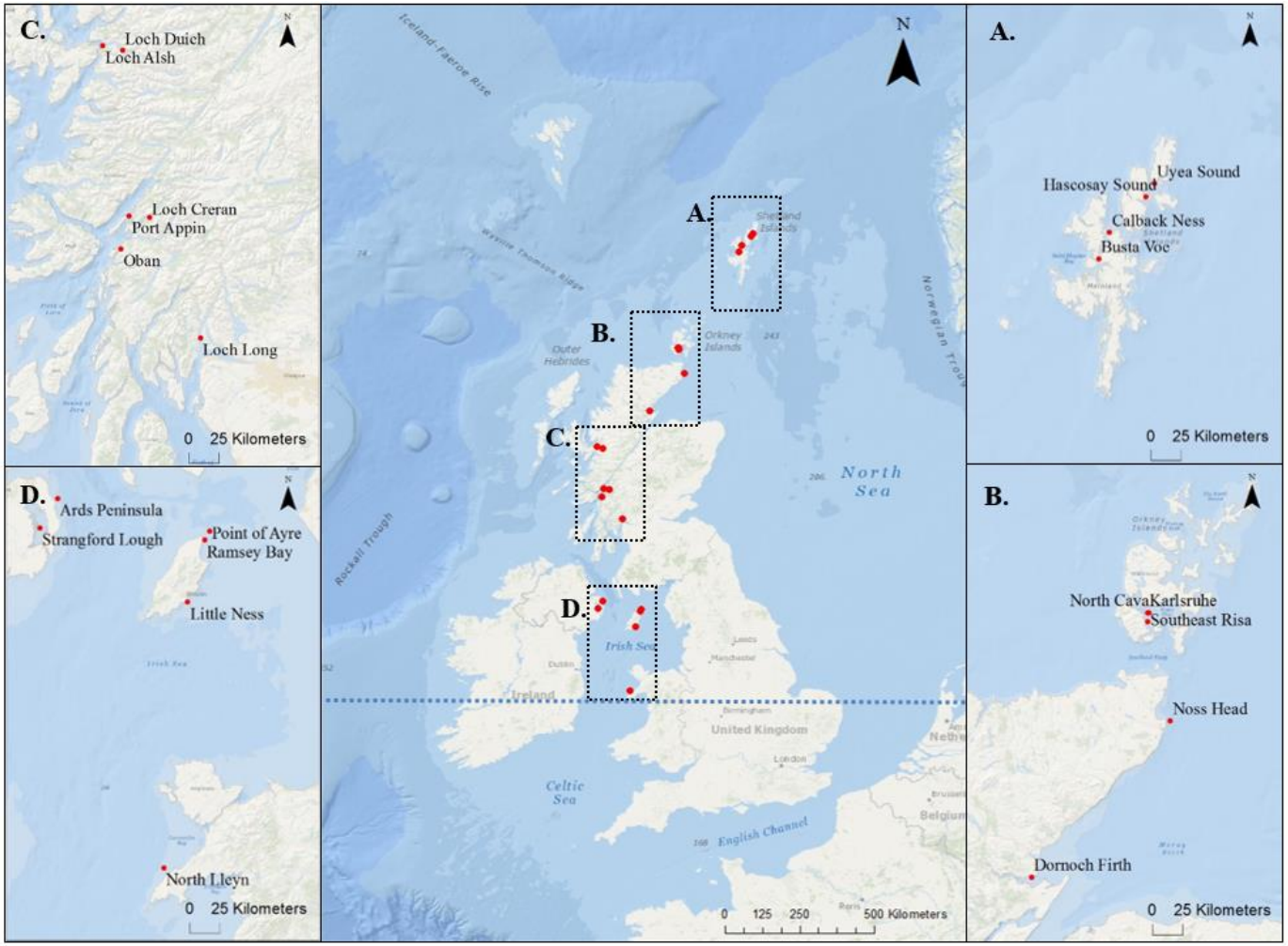


Figure 2.2. Distribution of *M. modiolus* reefs across the UK and Isle of Man. Dashed boxes within central map indicate extent of smaller inset maps (A-D). Site names provided within inset maps. Blue dashed line indicates approximate southern limit of *M. modiolus* reef habitats.

Historical Conditions

Temperature

Figure 2.3 demonstrates broad-scale seasonal temperature trends across coastal waters of the UK and Isle of Man according to NOAA data. Marine Scotland regional data for Scotland provide a comparison of temperature in coastal regions across mainland Scotland (Table 2.3, Figure 2.4). The Inner Moray Firth experienced the lowest (5.45°C) and highest monthly temperatures (13.14°C) across the year, therefore having the largest annual temperature range (7.69°C). Conversely, the Malin Shelf marine region has the smallest temperature range (3.88°C) but also experiences the highest minimum monthly temperature (8.39°C) across the year and highest mean annual temperature (10.12°C). Sullom Voe has the lowest maximum monthly temperature (11.14°C) and lowest mean annual temperature (8.27°C)

Table 2.3. Marine Scotland temperature data for Scottish regions containing or in proximity of *M. modiolus* reef sites. Monthly means (°C) and annual metrics (°C) are presented for seven regions of Scotland.

	Scottish Marine Regions						
Month	Clyde	Malin Shelf	North Minch	IMF	NC_N	NC_S	Sullom Voe
January	7.66	9.36	8.99	6.69	8.59	8.29	7.38
February	7.15	8.39	7.99	5.45	7.95	7.53	6.46
March	6.87	8.85	7.77	5.90	8.10	7.49	5.55
April	6.52	8.76	7.74	6.16	7.40	6.79	6.71
May	7.95	9.22	8.32	7.46	8.61	8.44	7.34
June	8.76	9.77	9.51	8.85	9.01	9.81	9.27
July	9.96	10.77	10.32	11.61	10.06	11.03	8.12
August	10.77	9.75	11.64	11.18	10.58	11.85	10.01
September	12.03	11.62	12.20	13.14	11.45	12.12	11.14
October	11.67	12.27	12.71	11.53	12.00	12.59	9.76
November	11.32	11.47	11.26	9.87	11.25	11.17	9.22
December	9.04	11.15	10.39	7.74	10.01	10.48	8.30
Annual metrics							
minimum	6.52	8.39	7.74	5.45	7.40	6.79	5.55
maximum	12.03	12.27	12.71	13.14	12.00	12.59	11.14
difference	5.51	3.88	4.97	7.69	4.60	5.80	5.59
mean	9.14	10.12	9.90	8.80	9.58	9.80	8.27

IMF=Inner Morary Firth, NC_N=North Coast (North); NC_S=North Coast (South)

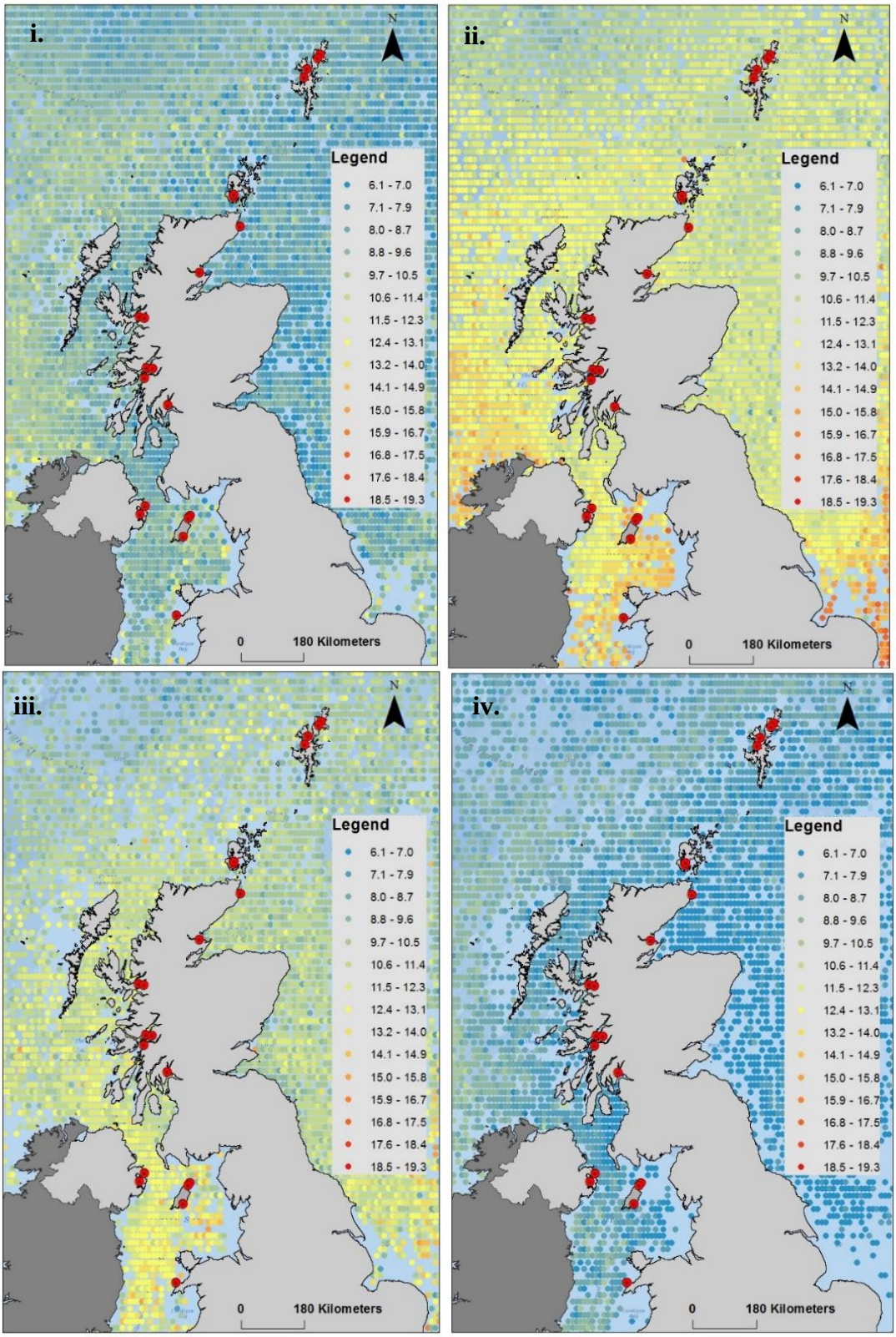


Figure 2.3. Broad-scale (i) spring, (ii) summer, (iii) autumn, and (iv) winter near bed temperature (NBT, approx. 25m) trends for the UK, Ireland and Isle of Man. Red dots indicate *M. modiolus* reef sites. 1° temperature data from NOAA (NOAA, 2016).

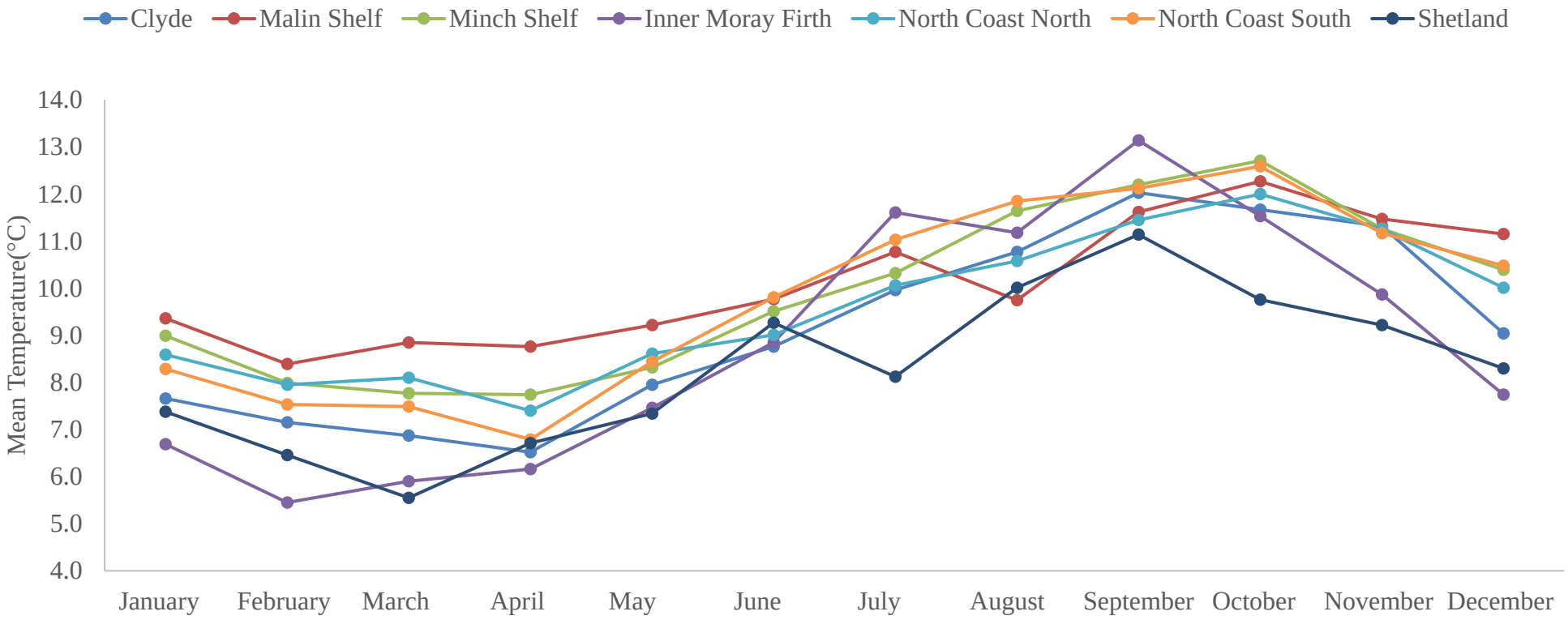


Figure 2.4. Annual Marine Scotland temperature data for Scottish regions containing or in proximity of *M. modiolus* reef sites (Source: Slesser &Turrell, 2013). Note: Data were derived by Marine Scotland from observations made by the Marine Laboratory, Aberdeen, during the period 1960-2010.

Temperature logger data showed latitudinal variation in annual temperatures across the North Lleyn, Loch Creran and Karlsruhe reefs (Table 2.4, Figure 2.5). The most southern site, North Lleyn, showed the highest mean temperature across all months (January-December) and across the year (11.86°C) followed by Loch Creran (9.96°C) and Karlsruhe (8.88°C). Across all months, North Lleyn was on average 1.91°C (±SD 0.45) warmer than Loch Creran and 2.99°C (±SD 0.48) warmer than Karlsruhe. Loch Creran was on average 1.08°C (±SD 0.47) warmer than Karlsruhe. North Lleyn showed the highest annual maximum temperature (16.5°C) followed by Loch Creran (14.02°C) and Karlsruhe (13.08°C) and also had the annual largest temperature variance (difference between maximum and minimum) (9.04°C) followed by Loch Creran (8.66°C) and Karslruhe (7.94°C).

Table 2.4. Annual temperature logger data for North Lleyn, Loch Creran, and Karlsruhe *M. modiolus* reefs sites including mean, minimum and maximum temperatures.

Month	**MEAN TEMPERATURE (°C)**			**TEMPERATURE RANGE (MIN, MAX) (°C)**					
	North Lleyn	Loch Creran	Karlsruhe	North Lleyn		Loch Creran		Karlsruhe	
January	9.43	7.95	6.88	8.96	10.18	7.13	8.77	5.86	7.78
February	8.65	6.65	6.01	8.12	9.15	6.02	7.12	5.55	6.88
March	8.09	6.48	5.82	7.46	8.77	5.36	7.25	5.14	6.37
April	8.67	6.92	6.24	8.21	9.43	5.90	8.26	5.45	6.98
May	10.81	8.33	7.80	9.24	11.88	7.51	10.33	6.88	9.08
June	12.74	10.53	9.10	11.69	13.68	9.17	11.72	8.68	9.77
July	14.78	12.36	11.51	13.80	15.80	10.85	13.88	11.04	12.98
August	15.84	13.47	12.43	15.30	16.30	12.99	14.01	11.43	13.08
September	15.88	13.55	12.37	14.73	16.50	13.06	14.02	11.72	12.88
October	14.64	13.23	11.05	13.87	15.11	12.44	13.74	10.06	11.82
November	12.38	10.77	9.50	10.65	14.06	9.25	11.98	8.68	10.26
December	10.47	9.27	7.79	8.59	12.35	6.98	10.64	6.78	8.88
ANNUAL	11.86	9.96	8.88	7.46	16.50	5.36	14.02	5.14	13.08

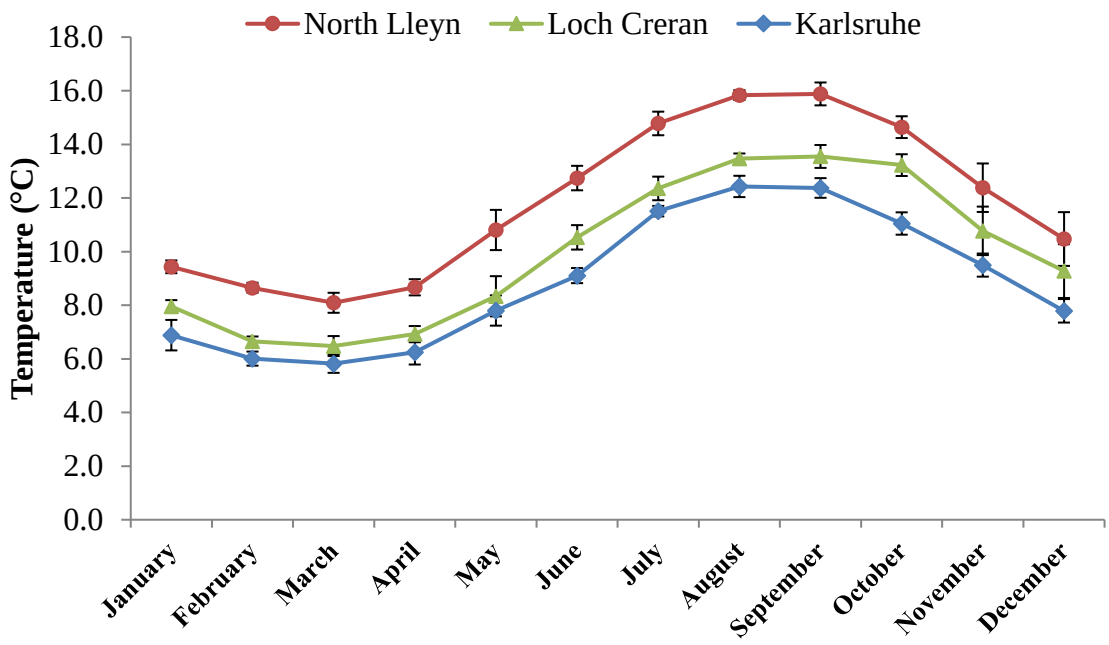


Figure 2.5. Annual temperature data for North Lleyn, Loch Creran, and Karlsruhe *M. modiolus* reefs sites. Mean values ±1SD (indicated by error bars) are presented for each month.

Dissolved Oxygen

Marine Scotland data provide a comparison of dissolved oxygen in coastal regions across mainland Scotland (Table 2.5, Figure 2.6). Sullom Voe marine region experienced the lowest (55%, in summer months) and highest (103%) monthly dissolved oxygen values across the year, therefore having the largest annual dissolved oxygen range (48%), and lowest mean annual dissolved oxygen content (80%). The Clyde and North Minch marine regions also had notable decreases in oxygen content (Clyde: 77%, North Minch: 75%) occurring during late summer-autumn.

Table 2.5. Annual dissolved oxygen (%) data for Scottish regions containing or in proximity of *M. modiolus* reef sites.

	Marine Area of Scotland						
Month	Clyde	Malin Shelf	North Minch	IMF	NC_N	NC_S	Sullom Voe
January	96	nd	89	95	nd	nd	nd
February	96	nd	86	93	nd	nd	nd
March	96	96	83	96	nd	nd	nd
April	94	98	80	nd	nd	98	103
May	95	95	103	nd	97	99	94
June	87	nd	102	nd	nd	103	81
July	82	94	100	nd	nd	nd	55
August	77	nd	nd	nd	nd	94	55
September	82	nd	75	85	92	93	nd
October	88	nd	95	nd	nd	nd	93
November	93	87	93	nd	94	95	94
December	97	nd	92	nd	nd	nd	nd
Annual metrics							
maximum	77	94	75	85	92	93	55
minimum	96	98	103	96	97	103	103
difference	19	4	28	11	5	10	48
mean	89	96	90	92	95	97	80

IMF=Inner Morary Firth, NC_N=North Coast (North); NC_S=North Coast (South); nd=no data

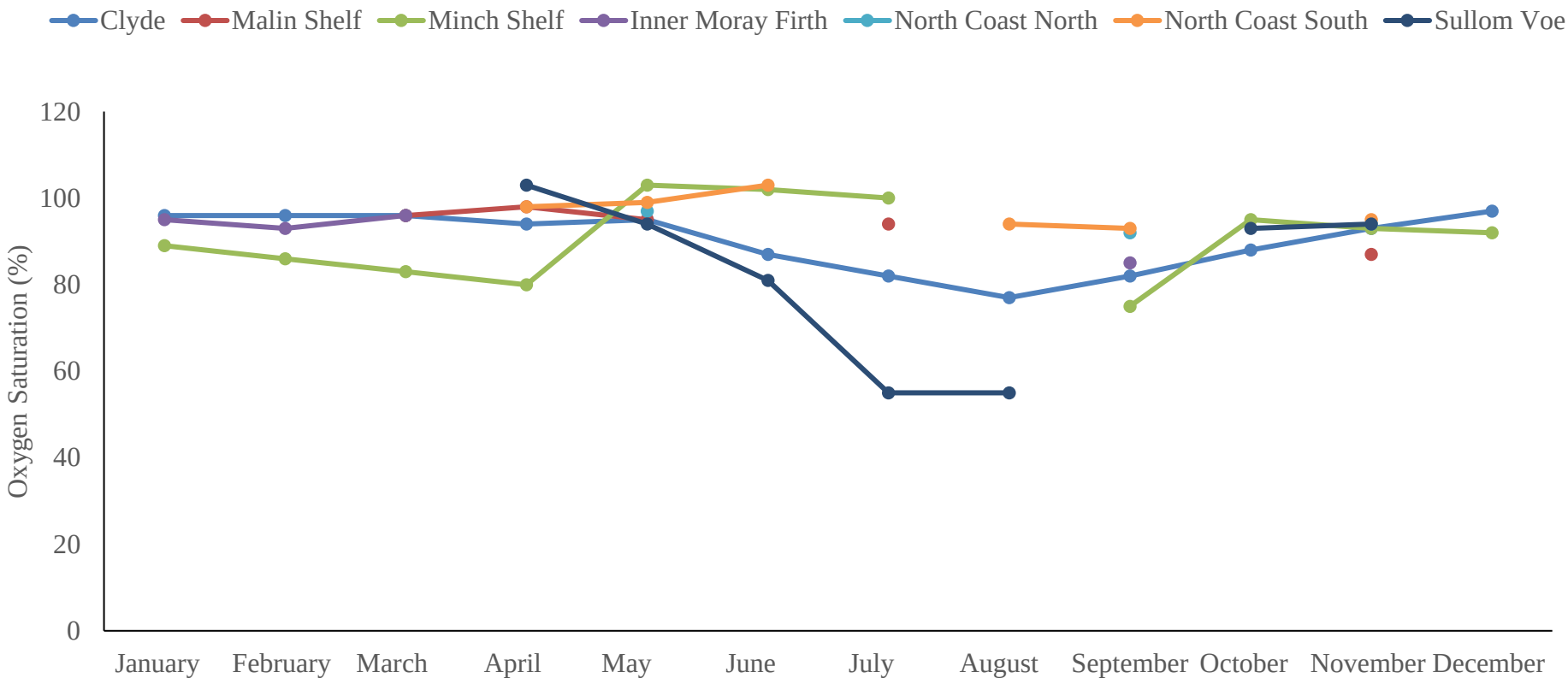


Figure 2.6. Annual dissolved oxygen (%) data for Scottish regions containing or in proximity of *M. modiolus* reef sites (Source: Slesser &Turrell, 2013).

Note: Data were derived by Marine Scotland from observations made by the Marine Laboratory, Aberdeen, during the period 1960-2010.

Climate Change Conditions

Temperature (Warming)

Figure 2.7 illustrates seasonal (spring, summer, autumn, winter) near bed mean temperatures expected under a medium emissions scenario for the period 2070-2099. Figure 2.8 indicates where seasonal near bed mean temperatures reach approximate thermal limits (15-20°C) for *M. modiolus*. These are only provided for Summer and Autumn seasons as such temperatures do not occur during Winter or Spring.

Figure 2.9 illustrates seasonal (Summer, Spring, Winter and Autumn) near bed maximum temperatures expected under a medium emissions scenario for the period 2070-2099. Figure 2.10 indicates where seasonal near bed maximum temperatures reach a range approximating approximate thermal limits (15-20°C) for *M. modiolus*. These are only provided for Summer and Autumn seasons as such temperatures do not occur during Winter or Spring.

Figure 2.11 shows how temperatures at specific sites (North Lleyn, Port Appin and Karlsruhe) could rise under the IPPC climate change projection of +4°C and indicates where temperatures move into the range approximating thermal limits (15-20°C).

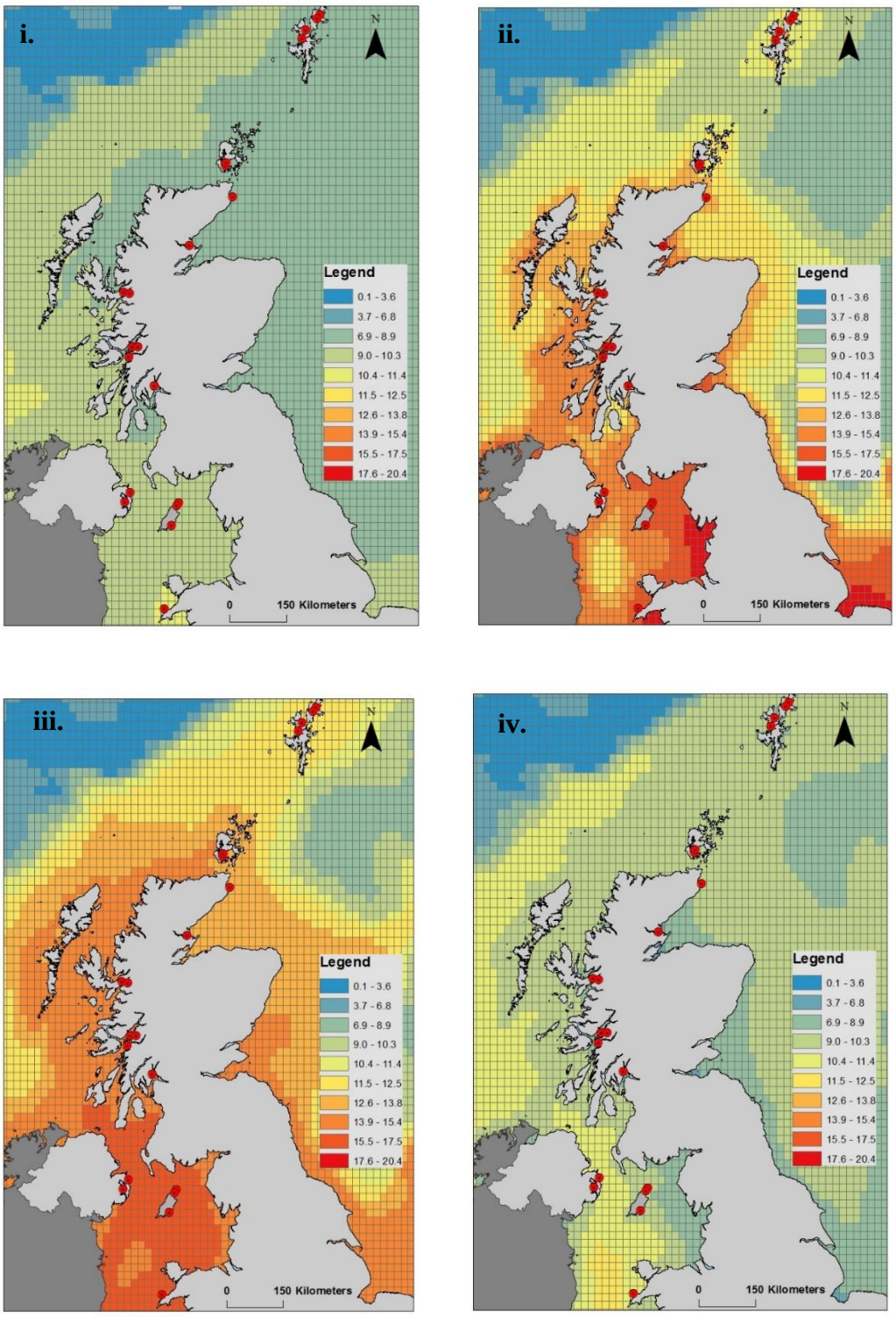


Figure 2.7. Mean near bed temperature under climate change (medium emissions scenario) for 2070-2099: (i) spring, (ii) summer, (iii) autumn, (iv) winter (Source: UKCP09 Projection Data).

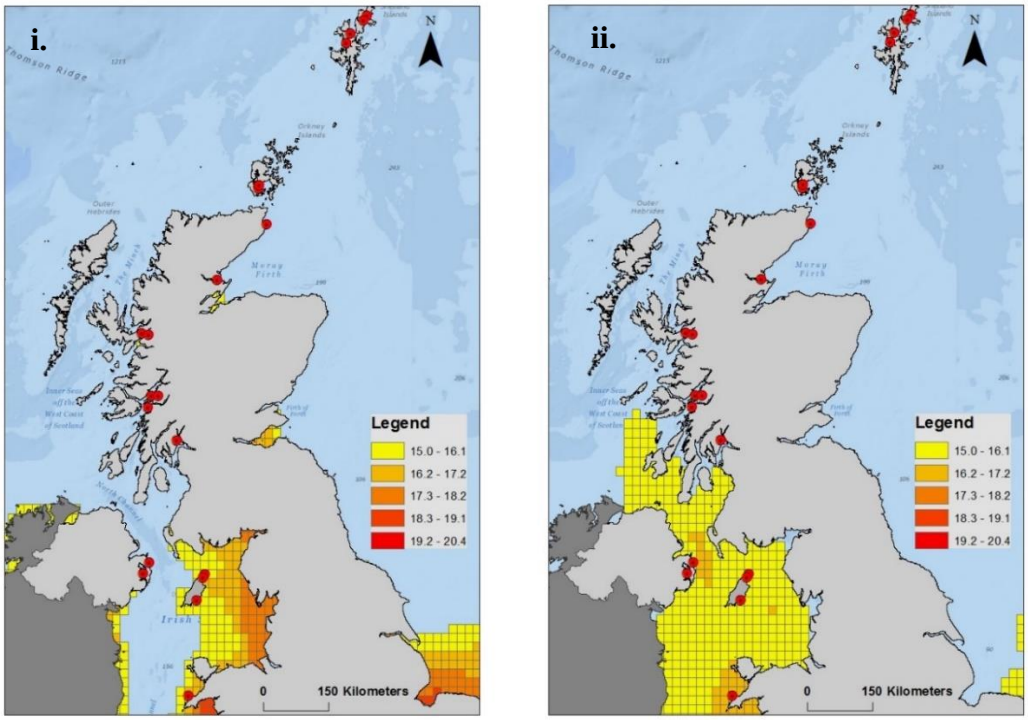


Figure 2.8. Mean near bed temperature > or equal to 15°C (*M. modiolus* thermal limits) under climate change (medium emissions scenario) for 2070-2099: (i) summer, (ii) autumn (Source: UKCP09 Projection Data).

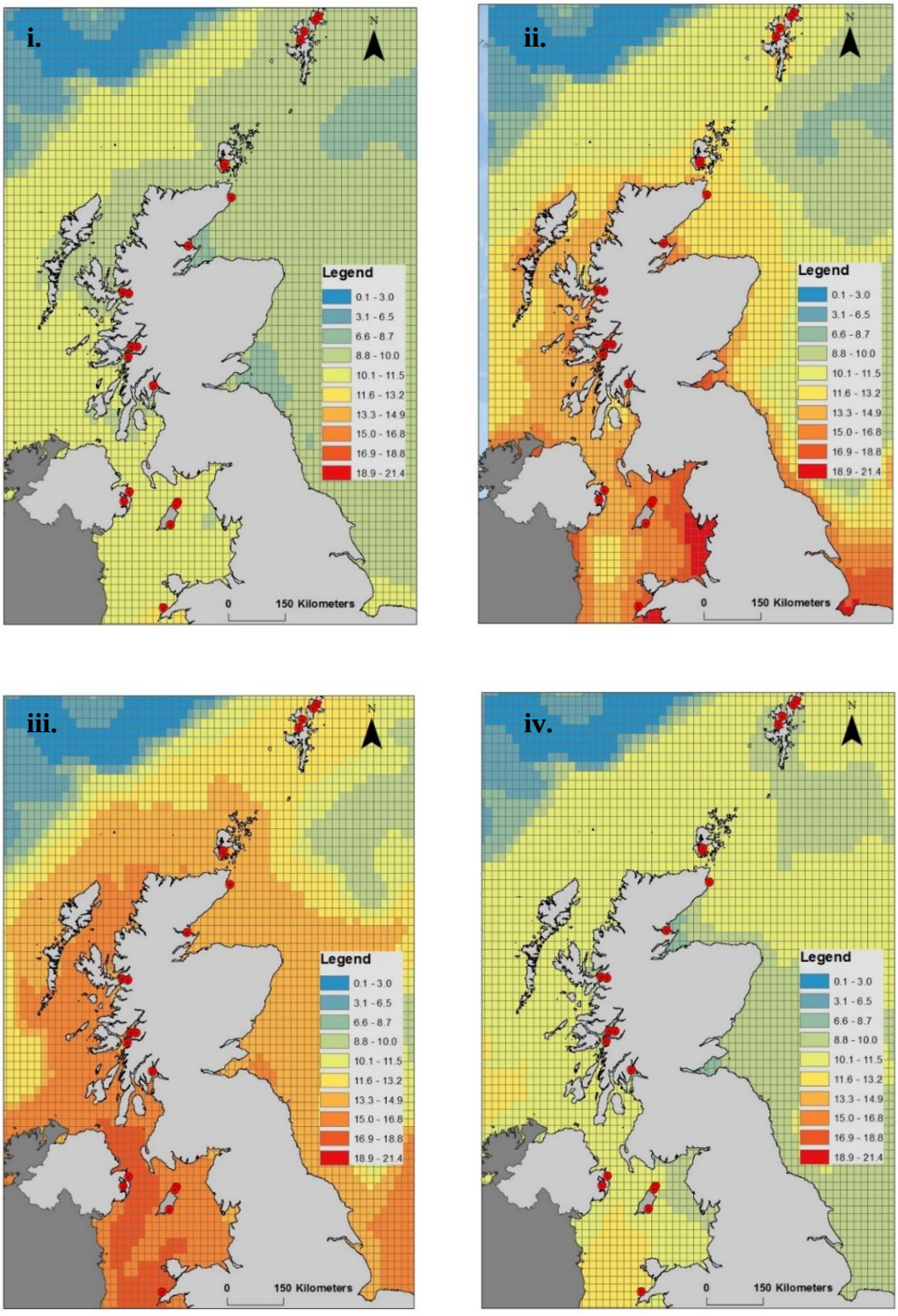


Figure 2.9. Maximum near bed temperature under climate change (medium emissions scenario) for 2070-2099: (i) spring, (ii) summer, (iii) autumn, (iv) winter (Source: UKCP09 Projection Data).

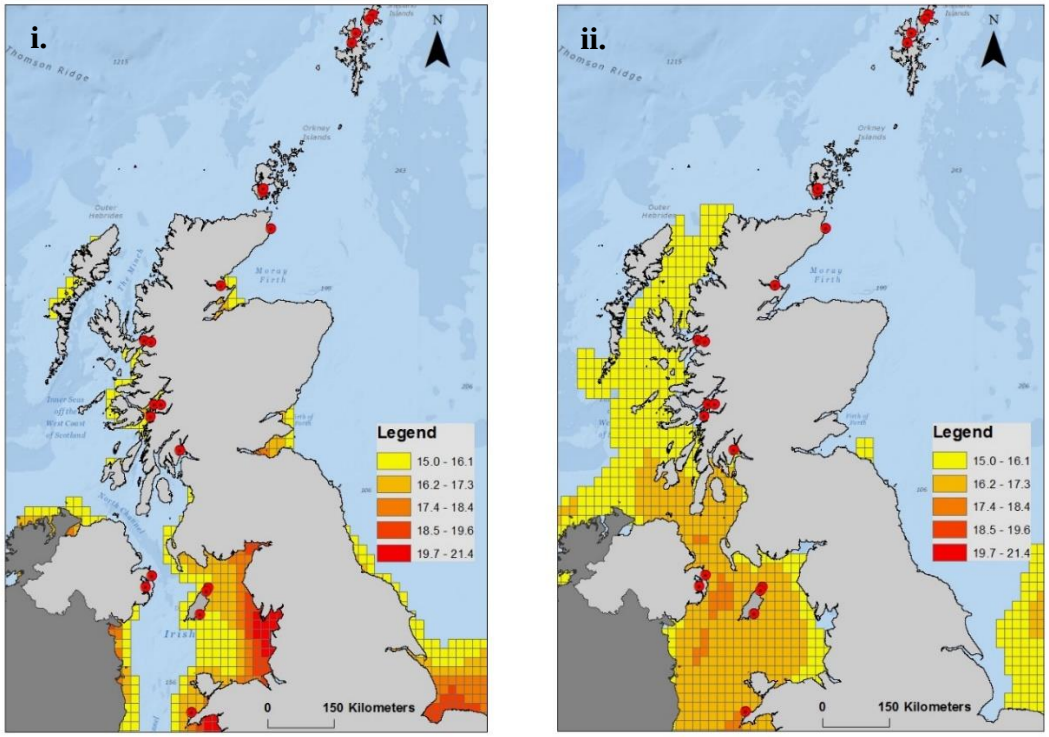


Figure 2.10. Maximum near bed temperature > or equal to 15°C (*M. modiolus* thermal limits) under climate change (medium emissions scenario) for 2070-2099: (i) summer, (ii) autumn (Source: UKCP09 Projection Data).

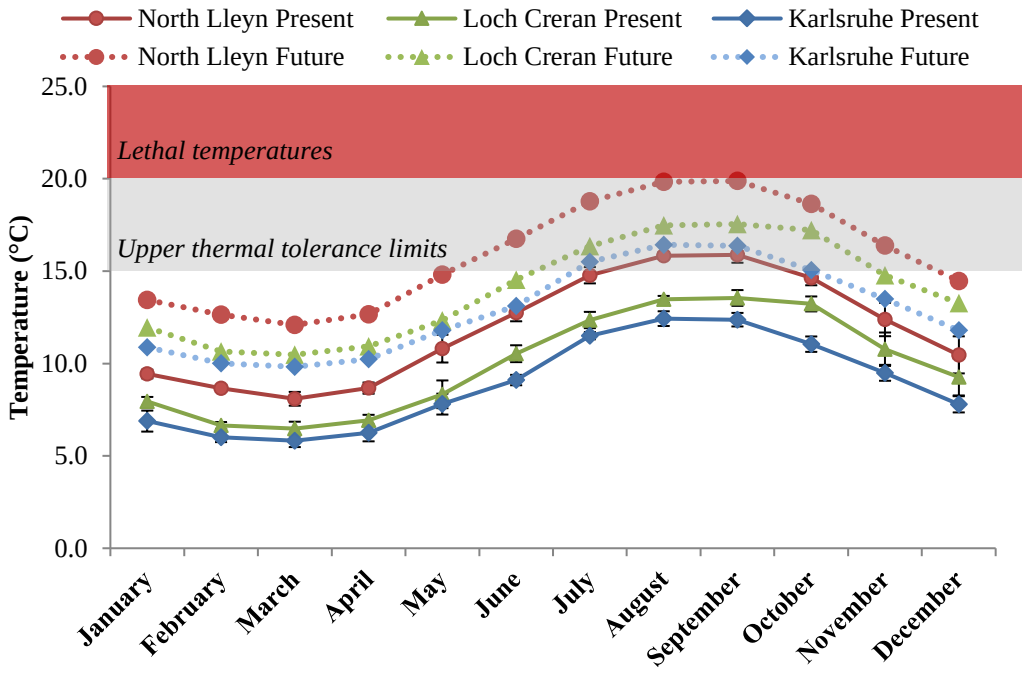


Figure 2.11. Climate projection of +4°C applied to annual temperature data for North Lleyn, Loch Creran, and Karlsruhe *M. modiolus* reefs sites. Present values displayed as means ±1SD (indicated by error bars) while future values equal present values +4°C. Grey shading indicates the proposed upper thermal tolerance for *M. modiolus* (Halanych *et al.*, 2013). Red shading indicates lethal temperature range (>20°C) for the species.

2.6 Discussion

Distribution

Distribution data indicate that *M. modiolus* (species) exist along much of the UK's coastline. However, greater densities exist in Scotland off the mainland west coast, and in Orkney Islands and Shetland Islands. Not surprisingly, the majority of reef sites also exist off Scotland's mainland west coast and in Orkney Islands and Shetland Islands. Similarly, Mair *et al.* (2000) reported a total of 67 Scottish sites where the Marine Nature Conservation Review (MNCR) database (JNCC, 2017) indicated "abundant" or "superabundant" *M. modiolus*, used as a proxy for potential reefs, with most sites falling in Shetland and northwest Scotland. Lancaster *et al.* (2014) also reported that Scotland supports 85% of all UK reefs. Notable populations also exist in Wales (North Lleyn), the Isle of Man (Point of Ayre, Ramsey Bay, Little Ness) and Northern Ireland (Strangford Lough, Ards Peninsula). While the relatively widespread UK distribution indicates that *M. modiolus* populations are capable of living under a range of conditions, the

accumulation of reef habitats along the Scottish west coast and northern isles (Orkney, Shetland) would suggest that conditions there are highly suitable for the habitat.

It should be noted that there is considerable uncertainty in the current distribution of *M. modiolus* reefs and it remains difficult to predict where reefs occur due to their relative inaccessibility (i.e. subtidal) and discrepancies between historical records and ground-truthing (Rees, 2009). Additionally, some *M. modiolus* reefs should be regarded as relict reefs as they may have established under different environmental conditions from those currently experienced. Where conditions have changed the key factors for successful establishment (e.g. depth, current flow), such reefs may be existing under sub-optimal conditions (Rees, 2009).

New reefs do continue to be found as a result of enhanced scanning technologies (e.g. Lidar, Sonar), dedicated survey effort and improved climate envelope analyses for the species. Climate envelope models use a species' current distribution to determine its environmental niche (e.g. temperature range) and then apply that information to spatially predict where the species will exist under current or future conditions (Sanford & Kelly, 2011). For example, Gormley *et al.* (2013) used the current temperature niche of *M. modiolus* to conclude that 100% of "suitable habitat" for the species would be lost by the year 2080. However, many climate envelope models assume that all populations have the same temperature limits and fail to consider populations where tolerances may differ from the species as whole (Sanford & Kelly, 2011). Consequently, consideration of regional/site-based differences, as highlighted here, and differences in population tolerances (e.g. thermal limits, see Chapter 4) may contribute to an improved understanding of the climate envelope for *M. modiolus* reef habitats.

Historical Conditions

Temperature

An understanding of how environmental conditions have shaped the current physiology of organisms can aid in predicting how climate change will affect those organisms' future physiology (Hofmann & Todgman, 2010). Consequently, examination of thermal histories of *M. modiolus* populations across the UK can shed light on how these populations may cope under warming conditions. Temperature data indicate that *M. modiolus* reefs across the UK range of the habitat experience a wide array of temperatures

depending on location and time of year. Comparison of all populations demonstrates a clear latitudinal gradient in temperature (i.e. decrease) with north-south movement approximating an annual temperature range of 5-17°C. NOAA data, for example, show that during summer months, temperatures around far northern (Orkney and Shetland Islands), northern (Scottish mainland) and southern (Isle of Man, Wales, Northern Ireland), populations range from approximately 9-13°C, 12-15°C and 12-17°C, respectively. Likewise, site specific temperature data for North Lleyn, Loch Creran and Karlsruhe reef sites indicate that the North Lleyn is substantially (2-3°C) warmer across the year than northern conspecifics. Further, the North Lleyn population has a large portion of the year (July-September) where it experiences temperatures within the upper thermal tolerance range for the species (i.e. 15-20°C).

There is also considerable variation in temperature variability across sites. Marine Scotland regional data for Scottish sites, for example, suggest that the Dornoch Firth reef population experiences the most extreme and variable temperature conditions of all Scottish sites. Data also suggest that Oban, Port Appin and Loch Creran populations experience less temperature variability but, on average, warmer temperatures across the year than any other population. Unsurprisingly, the dataset also suggests that Shetland populations (Caldback Ness, Hascosay Sound and Uyea Sound) live in lower temperature environments for most of the year as compared to other southern sites. Additionally, site-specific data indicate that the most southern *M. modiolus* reef (North Lleyn) has greater variation in temperature than more northern populations (Loch Creran and Karlsruhe). It should also be noted that in Scotland, *M. modiolus* reefs occur across a wide range of depths from as shallow as 5m up to 250m (Mair *et al*., 2000) and populations will therefore experience wide variation in temperature ranges and extremes according to depth.

Dissolved Oxygen

Marine Scotland regional data for Scottish sites indicates that Shetland populations (Calback Ness, Hascosay Sound and Uyea Sound) experience lower oxygen conditions during summer months (August, September). Though to a lesser extent, the dataset also suggests that populations along the west coast (Loch Long, Loch Duich and Loch Alsh) experience lower oxygen conditions during late summer/autumn (August, September).

Hypoxia has also been documented in Sullom Voe (Shetland Islands) which is seasonally subjected to low oxygen conditions (Gray *et al*., 2002; Mair *et al*., 2010).

The key cause of hypoxia or anoxia is eutrophication events, primarily caused by discharge of nitrates and phosphates into coastal waters (GESAMP, 2001). Eutrophication occurs when such increases in input of nutrients and dissolved organic matter (DOM) into the water column cause increases in particular organic matter (POM) originating from bacteria, phytoplankton and zooplankton (i.e. algal blooms). The excess organic matter cannot be dealt with via normal grazing activity and instead sinks to the seabed where degradation quickly decreases oxygen levels. Consequently, benthic sessile marine organisms such as *M. modiolus* are abruptly faced with low or no oxygen conditions (i.e. hypoxia or anoxia). The risk of hypoxia/anoxia is magnified in areas where there is little vertical mixing (e.g. low energy environments) and in proximity to high nutrient run-off from surrounding land mass (Gray *et al.*, 2002). For example, *M. modiolus* populations situated in fjords (e.g. Loch Creran and other loch populations of the west coast of Scotland) are at higher risk of hypoxia/anoxia as such areas are characterised by limited water exchanges in their bottom water and tend to be narrow channels surrounded by land mass (Gray *et al.*, 2002). Additionally, *M. modiolus* in these coastal loch areas can be situated near fish farms (characteristic of the region) and thus may be at threat of intermittent hypoxia brought on by increased fish waste (Rees, 2009).

Influence on Phenotypic Plasticity and Adaptation

The historical conditions experienced by populations influence both phenotypic plasticity (acclimatory ability) and local adaptation, and therefore shape the tolerances of resident populations. Genetic adaptation refers to a change in allele frequencies in a population via natural selection and is typically a slow (i.e. generations) and irreversible process. Plasticity, on the other hand, refers to variable expression of genetic trait in response to conditions and may be reversible or irreversible within an organism's lifetime (Helmuth *et al*., 2005). The difference is important as while adaptation represents a genetic tolerance that may be transferred to other sites via transplantation or larval delivery, phenotypic plasticity may be lost as the organism adjusts to new site conditions. However, it is extremely difficult to determine whether phenotypic plasticity or local adaptation is responsible for differences that may exist between populations, and

plasticity may limit adaptation to stress conditions by masking genetic variance (Sanford & Kelly, 2011).

Phenotypic plasticity in response to environmental variation is well documented in marine organisms with differences in exposure inducing modifications in morphology physiology and behaviour (Sanford & Kelly, 2011; Calosi *et al*., 2016). For example, *M. modiolus* populations showing variability in historical conditions or that exist despite experiencing thermal extremes (e.g. North Lleyn, Dornoch Firth) may be adapted to cope with increased stress conditions or may possess phenotypic plasticity to cope with their current conditions. Under lab comparison of thermal limits, populations experiencing thermal extremes in their natural environment have been shown to be more tolerant of higher temperatures than conspecifics that do not typically experience such extremes (Kuo & Sanford 2009). Therefore, *M. modiolus* that experience higher temperatures on a regular basis, may be better able to cope with warming conditions as compared to those accustomed to lower temperatures.

Climate Change Conditions

UKCP09 data indicate that under climate change warming conditions many *M. modiolus* reef sites will experience mean seasonal temperatures approaching thermal limits for the species, particularly in the southern extent of the distribution (i.e. Isle of Man, Wales and Northern Ireland populations) where mean summer and autumn near-bed temperatures could rise to approximately 17°C. Furthermore, when maximum near-bed temperatures are considered, most sites (except north-eastern Scotland, Orkney Islands and Shetland Islands) experience temperatures greater than 15°C especially during the autumn season. Application of IPCC temperature projection to site data for North Lleyn, Loch Creran and Karlsruhe populations shows that all three populations will experience temperatures in the upper thermal tolerance range for *M. modiolus* (i.e. 15-20°C) but length of exposure varies according to site. Notably, the North Lleyn population may experience temperatures in the upper thermal tolerance range for more than half the year (May-November) with August and September months reaching as high as 20°C. Therefore, this population appears to be at considerable risk under climate change conditions. Moreover, the more northern sites are also at risk with Loch Creran population potentially experiencing temperatures as high as approximately 18°C (September) and Karlsruhe potentially experiencing temperatures as high as approximately 16.5°C (August).

Populations existing in shallow coastal or enclosed sea lochs will also be at increased risk of hypoxia due to strengthened thermal stratification under warming conditions, and hypoxic events may also extend into previous unimpacted coastal areas. Additionally, hypoxic events may be exacerbated by coinciding climate-induced changes including increases in freshwater discharge and nutrient loads (leading to increased primary production; i.e. algal blooms) (Rabalais *et al*., 2010; Birchenough *et al*., 2015). Consequently, it is likely that populations previously shown to experience hypoxic conditions (e.g. Shetland and Scottish west coast populations) will be at increased risk under climate change. Additionally, it is proposed that all coastal *M. modiolus* populations will have some increased risk of hypoxia under climate change conditions.

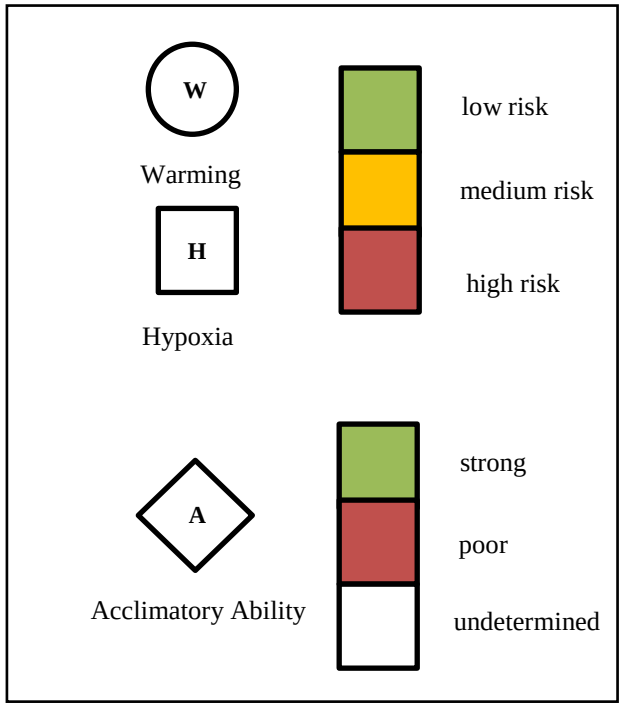
Assessing Vulnerability

The task of determining vulnerable or resilient populations according to environmental conditions is challenging. However, knowledge of past and future conditions across sites can at minimum aid in assessment of population-based vulnerability to changing conditions. Data suggest that the most southern population, North Lleyn, is under high threat of warming and one would also expect other southern populations at similar depths to North Lleyn (i.e. Isle of Man) may also experience thermal stress. However, given the North Lleyn's thermal history (high variability and reaching thermal maxima in summer months), the population may have some acclimatory capacity. Further, if adaptation has taken/does take place, the population may be valuable in providing more resilient larvae to northern populations (see Chapter 3, pp. 59-111, for investigation of population connectivity). Other populations where variability of conditions is considerable (e.g. Dornoch Firth) may also demonstrate some degree of acclimation/adaptation to climate change. Conversely, populations along the west coast of Scotland appear to experience a lower degree of variability of temperature conditions and thus may struggle with changing conditions. Additionally, these populations may be under greater threat of hypoxia due to the geography of the area (i.e. narrow channels with limited water exchange potentially in proximity of nutrient input from land mass and fish farms) and this threat will increase under climate change. Populations living at greater depth (i.e. buffered stable environments) such as the Noss Head population may be protected from more immediate climate change but in the long-term are likely to struggle to cope with changing conditions. Table 2.6 summarises the influence of physical conditions to the vulnerability and Figure 2.12 illustrates potential vulnerabilities of populations across the

UK range based the above discussion. Next, other key factors may be considered as additional influences on the vulnerability of *M. modiolus* populations to climate change. These include population genetics (Chapter 3) and acclimatory ability (Chapters 4-5).

Table 2.6. Influences on *M. modiolus* vulnerability under climate change conditions of warming and hypoxia

		Vulnerability	
Climate change	**Influence**	HIGH ⟷	LOW
Warming	Latitude	Southern (higher temperatures)	Northern (lower temperatures)
	Depth (degree of warming)	Shallow (higher temperatures)	Deep (lower temperatures)
	Depth (acclimation/adaptation to conditions)	Deep (historically stable conditions)	Shallow (acclimated/adapted to variable/extreme conditions)
	Historical variability	Low variability (narrow thermal tolerance range)	High variability (wide thermal tolerance range)
Hypoxia	Latitude	Southern (warmer temperatures = less oxygen, increased frequency of eutrophication)	Northern (cooler temperatures = increased oxygen)
	Location	Coastal (nutrient influx from land mass, salmon farms, etc.)	Offshore (reduced nutrient influx)
	Exposure	Enclosed (low energy = little mixing)	Tidally-swept (high energy = mixing)



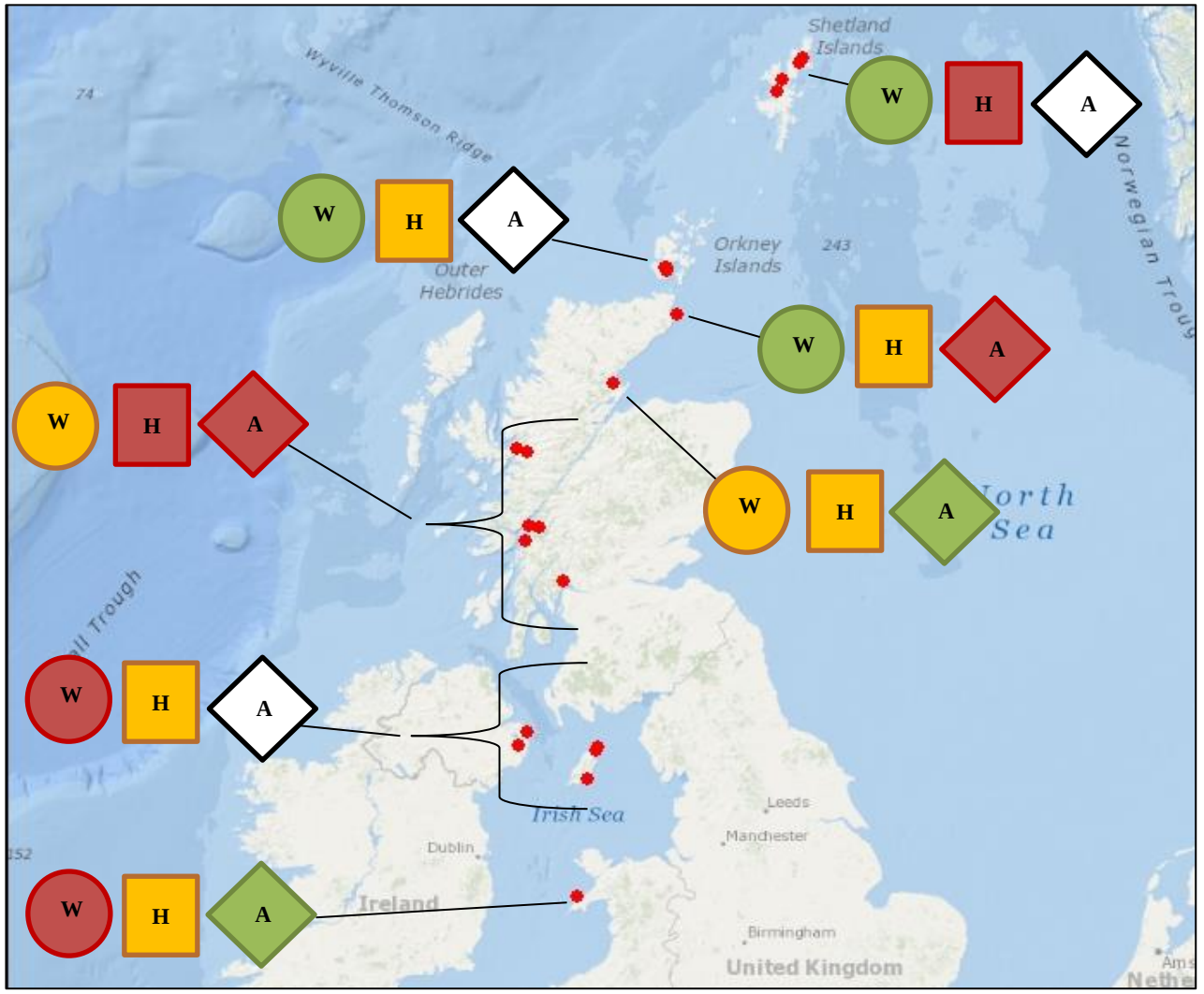


Figure 2.12. Potential vulnerabilities of *M. modiolus* populations across the UK range based on risk of warming and hypoxia, and potential acclimatory ability (according to thermal history of site and variability of conditions).

3. **Adaptive Capacity and Connectivity:** Genetic structure of UK *M. modiolus* reefs

3.1 Abstract

Spatial variation in conditions across the range of *M. modiolus* reef habitats results in spatially distinct populations existing under unique sets of environmental conditions. Consequently, populations may be faced with varying selective pressures that give rise to genetic adaptations for coping with environmental stressors. Conversely, the movement of genetic material (i.e. gene flow) between populations may reduce genetic adaptive capacity but lead to increased genetic connectivity and diversity of populations. This relationship between genetic adaptation and gene flow results in differing levels of shared genetic makeup and shifting degrees of resistance to environmental stressors across populations. In this chapter, the genetic structure of *M. modiolus* reefs from across the UK and Isle of Man (IOM) was investigated via microsatellite screening to ascertain the connectivity of reefs and to examine potential adaptive capacity of populations under climate change conditions. Populations were screened to examine broad-scale and regional patterns of connectivity and diversity with results providing key information for effective management of *M. modiolus* reefs (e.g. the role of MPAs, transplant suitability for restoration efforts). Results indicated moderate to strong levels of connectivity across the network, hypothesised to be a result of hydrodynamics, demographics and site geography but unrelated to distance. Data shows two key structural genetic groups across Scottish and IOM regions but generally comparable levels of migration within and between regions. Similarly, equivalent levels of genetic diversity were detected across populations. Results are discussed in relation to climate change vulnerability and management.

3.2 Aims and Objectives

Aims:

- Determine the genetic connectivity of *M. modiolus* populations from across the species' UK range to illustrate the general structure of the UK network;

- Illustrate the potential adaptive capacity of *M. modiolus* populations under climate change conditions via examination of population genetics (e.g. genetic diversity indices and genetic connectivity);

- Apply genetic tools to generate data which can be used to inform management of *M. modiolus* biogenic reef sites

Key Objectives:

- **Investigate genetic connectivity and diversity** of *M. modiolus* populations across the UK range of the species via microsatellite screening;

- Use microsatellite screening to **determine transplant suitability** for restoration of a degraded *M. modiolus* reef habitat (Strangford Lough, Northern Ireland);

- Use microsatellite screening to **examine genetic connectivity, genetic structure and migration** in Scottish and Isle of Man *M. modiolus* populations in order to consider the role of Marine Protected Areas to wider regional *M. modiolus* networks.

- **Discuss the role of observed genetic connectivity and diversity in shaping vulnerability** of *M. modiolus* under climate change conditions.

3.3 Introduction

When the distribution range of a species is spread across a diverse landscape, spatial variation inevitably leads to spatially distinct populations inhabiting areas with a particular, and often unique, set of parameters. Accordingly, intraspecific populations existing under different conditions may develop varying tolerances to environmental stressors. Variation in tolerance may be due to phenotypic plasticity, genetic effects (e.g. adaptation), or, as is most usual, a combination of both factors (Luttikhuizen *et al.*, 2003).

Genetic effects result when spatial variation in conditions exerts selective pressures upon a population. This leads to both local adaptation within the given population and increased genetic differentiation from other populations (Whitlock, 2008). While spatial heterogeneity can lead to genetic variation, gene flow driven by dispersal has the opposite effect. Gene flow refers to the movement of genetic material (via migration or larval movement) from one population to another, and results in populations with a shared genetic makeup and therefore reduced genetic differentiation. Consequently, gene flow has a large influence on both inter- and intra-population levels of genetic diversity and connectivity. Gene flow and adaptation are not exclusive, and populations are often influenced by both mechanisms. Inevitably, the interplay of these genetic drivers leads to varying levels of shared genetic material and shifting degrees of resistance to environmental stressors across populations (Whitlock, 2008).

Marine bivalves characteristically have high fecundity and release larvae with high dispersal potential, resulting in species with substantial geographic ranges, large population sizes and high rates of gene flow between populations (Luttikhuizen *et al*., 2003). Previously, it has been largely assumed that the marine environment poses few absolute barriers to gene flow so that populations that are widely separated may be genetically very similar. Consequently, it has been suggested that many marine species have little genetic population structure and rather, act as large panmictic units with low rates of allopatric speciation (Palumbi, 1994). However, oceanic processes including currents and tidal flows, seasonal front systems (e.g. thermoclines) and boundary layers may promote gene flow between areas or may alternatively act as invisible barriers which restrict or hamper larval movement including imposing seasonal and directional limitations (Hohenlohe, 2004; Robins *et al*., 2013). Additionally, such forces directly

influence the availability and species composition of planktonic communities which, as a key food source, further shape the dispersal success of larvae (Palumbi, 1994).

The determination of genetic connectivity of marine species is an important contribution to understanding climate change vulnerability of that species. Low levels of connectivity (i.e. high genetic differentiation) drive local adaptation among populations and are therefore a large influence on the ability of populations to adapt to changing conditions (Harley *et al.*, 2006). This is particularly important for populations that experience increased stress conditions (as compared to cohorts) and must adapt or perish. On the other hand, high levels of population connectivity allow genetic material to be shared with isolated or sink populations and are therefore critical to maintaining a network of populations. In either case, determination of population connectivity has been advocated as a critical component towards effective protection and management of a species of high conservation importance such as *M. modiolus* (Weersing & Toonen, 2009; Lowe & Allendorf, 2010).

Measuring Spatial Variation in Genetic Structure

Recent and ongoing advances in the field of genetics have resulted in the development of a suite of genetic tools which are instrumental in analysing spatial variation in genetic structure and may be used to examine demographics between populations, to understand genetic structure across populations, and to provide evidence of population-based differences. Marker types include allozymes, mitochondrial DNA, single nucleotide polymorphisms (SNPs) and microsatellites (Hellberg *et al*., 2002; Wan *et al*., 2004). The current research focused exclusively on microsatellite markers for examining *M. modiolus* population genetic diversity and connectivity.

Microsatellite markers have gained popular use for determination of genetic structure in eukaryotic nuclear genomes. Specifically, microsatellites refer to deoxyribonucleic acid (DNA) sequences of variable length which are repeated in tandem through the genome and have a high rate of mutation (e.g. are polymorphic). They hold the benefits of allowing identification of both homozygote and heterozygote genotypes and being relatively simple and low-cost to run via PCR methodology (Selkoe & Toonen, 2006; Hoshino *et al.,* 2012). Additionally, microsatellite markers provide high information content and are incredibly versatile with regards to their ability to answer numerous types

of ecological questions, with applications including bioinvasion and epidemiology studies, genome mapping, genetic diversity analyses and investigation of population and phylogenetic relationships (Hoshino *et al*., 2012). Nonetheless, there are a number of limitations to this type of marker including (i) issues around homoplasy (i.e. alleles identical in length but not by descent) leading to underestimation of actual divergence between populations; (ii) the presence of null alleles (i.e. where locus deletion or mutation in the annealing primer site prevents locus amplification) leading to inaccurate estimation of allele frequencies and segregation rates; and (iii) inconsistent allele size calling resulting in inaccurate data (Hoshino *et al*., 2012). Consequently, Single Nucleotide Polymorphisms (commonly referred to as SNPs) markers are increasingly being looked to as the genotyping tool of choice over microsatellites. However, SNPs require full genome sequencing which is yet to be completed for *M. modiolus*, and therefore this marker type remains unavailable for the species (Zhang & Hewitt, 2003).

Application of Population Genetics to Management

Management of priority marine habitats of high conservation important such as *M. modiolus* may benefit from detailed knowledge regarding population genetic structure and differentiation. Such information may be of use in informing restoration of degraded habitats and management of Marine Protected Areas (MPAs), discussed as follows.

Restoration Efforts

In Northern Ireland, particular focus has been provided to Strangford Lough where *M. modiolus* reefs have declined in distribution, extent, condition and density since the mid-1970s (Roberts *et al*., 2004, 2011). Until such time, reefs were found extensively throughout the area and were associated with high levels of biodiversity (Roberts *et al*., 2004, 2011). Currently, efforts are underway to restore the Strangford Lough *M. modiolus* reefs with recent recommendations including the transfer of adult mussels to areas characterized as having high potential for successful translocation (i.e. according to habitat suitability, larval dispersal modelling and historical distribution of the species) and falling within a proposed non-disturbance zone. However, given that translocation requires acquiring sufficient number of *M. modiolus* and animals would need to be collected from outside the area, the issue of introducing genetically non-compatible animals has been raised (Roberts *et al*., 2011). Given its close proximity and condition, the Ards Peninsula *M. modiolus* population has been proposed as a prospective source of

M. modiolus individuals for translocation to Strangford Lough. To support restoration efforts, the current study aimed to measure genetic differentiation between the two populations in order to determine transplant suitability.

MPA Management

In Scotland, several *M. modiolus* biogenic reef sites fall within MPA-designated waters, including populations in Shetland Islands, Noss Head off mainland Scotland's north coast, Loch Creran on the west coast and Dornoch Firth on the east coast. Under Scottish Natural Heritage (SNH), it is of considerable conservation importance and priority to determine the role that these populations play in the wider system of *M. modiolus* reefs across Scotland and the UK. For example, MPAs acting as source populations for other populations will be of substantial value to maintaining a viable network. Likewise, those populations acting as sink populations are important settlement sites for *M. modiolus* larvae and thereby essential for recruitment. Clarification of such relationships will help to determine the extent to which MPAs act independently/dependently with other MPA sites and with populations falling outside of such areas. Such information will contribute to the development and achievement of conservation objectives for MPAs, highlight the value of MPAs to the wider network of populations, and ultimately, aid in the preservation of these valuable habitats.

Similarly, the Isle of Man Government (IOM) Department of Environment, Food and Agriculture (DEFA) has recently designated an Isle of Man *M. modiolus* reef site known as the Little Ness site, situated south of Douglas along the south-eastern coast of the IOM. There is considerable interest in the relationship of the newly protected site to other local populations (including Ramsey Bay MPA and Point of Ayre sites) of the Isle of Man. For example, analyses of the Little Ness and Point of Ayre populations could potentially provide useful information on how hydrodynamics of the area might influence genetic connectivity across more localised IOM sites and at a broader scale of the Irish Sea (e.g. connectivity of southern and northern Irish Sea populations).

This chapter provides an overview of research that was carried out to investigate genetic structure of UK and IOM populations for (i) defining the genetic connectivity of the *M. modiolus* network at varying spatial scales (local, regional, UK-wide); (ii) determining genetic diversity of populations; and (iii) determining the role of populations within

MPAs. An initial investigation was conducted with a small number of key UK *M. modiolus* populations via application of a preliminary set of microsatellite markers (Gormley, 2014) and included a focused analyses of Irish Sea populations, carried out in collaboration with Dr. Kate Gormley. Results aided in validating the marker set and contributed to a number of outputs including application of connectivity analyses for determination of transplant suitability in Northern Ireland populations and publication of Irish Sea *M. modiolus* genetic connectivity results in a peer-reviewed journal (Gormley *et al.*, 2015).

Following the success of the initial investigation, a further number of microsatellites were developed (externally) for undertaking a more robust and focused connectivity and diversity analysis with direct application to management. This enhanced investigation paid particular focus to the connectivity and diversity of reefs within the MPA network and also provided information regarding the relationship between reefs within MPAs and those external to such areas. This work was funded by and carried out for (i) SNH, with specific regard to Scottish reef populations; and (ii) the Isle of Man (IOM) Government, with specific regard to IOM reef populations.

3.4 Methodology

Part 1: Preliminary Microsatellite Screening of UK Populations

Animal Collection

M. modiolus were hand collected by the Heriot Watt Scientific Dive Team from six sites distributed across the UK distribution of the habitat including the southern limit (North Lleyn) and northern extent (Orkney) (Table 3.1, Figure 3.1). Animals were transported in cold boxes (with seawater and ice packs) to aquaria at Heriot-Watt University and kept in large flow- through tanks (~15^0C) prior to processing.

Table 3.1. Collection data for preliminary microsatellite screening of UK *M. modiolus* populations.

Site Name (Region)	Date	# of samples	Latitude	Longitude	Depth (m)
North Lleyn (Wales)	June 2010	50	-4.654	52.944	32.7
Point of Ayre (Isle of Man)	nd	31	-4.305	54.439	nd
Ards Peninsula (Northern Ireland)	nd	50	-5.457	54.582	nd
Strangford Lough (Northern Ireland)	April 2011	50	-5.594	54.454	24.9
Port Appin (Western Scotland)	Nov 2011	50	-5.424	56.551	24
Karlsruhe (Orkney Islands)	Sept 2011	50	-3.19	58.889	nd

nd=no data

Figure 3.1. Map of UK and IOM *M. modiolus* reef locations screened with microsatellite markers for preliminary analyses of genetic connectivity and diversity.

Sample Preparation

Adductor tissue was dissected from each sample, cut into several smaller pieces, and preserved in approximately 15-20 mL 96% ethanol solution in 25 mL specimen tubes. Samples were stored at 4°C prior to DNA extraction.

DNA Extraction

DNA extractions were carried out with Qiagen DNeasy Blood & Tissue Kits (Qiagen, Manchester, UK) according to the Qiagen protocol (Qiagen, 2006). Approximately 2-3g of preserved adductor tissue was removed from ethanol, and cut into small pieces on a sterile tile surface using a sterile scalpel blade. All surfaces and utensils were cleaned with ethanol prior to the start of individual sample processing. Genetic material was transferred to a sterile 1.5 mL centrifuge tube. Next, 180 ul of ATL buffer (lysis buffer) and 20 uL of proteinase K were added and the sample was then vortexed before incubation at 56°C for 1 hr. Samples were re-vortexed after 30 minutes of incubation. Following the incubation period, 200 uL of AL buffer was added to each sample and samples were re-vortexed. Next, 200 uL of ethanol was added to each sample and samples were re-vortexed to allow for precipitation of DNA. All liquid was then removed from each centrifuge tube and pipetted onto the filter of a centrifuge column (with collecting tube) before centrifugation for 1 minute at 8000 rpm. Following, the column was placed into a new collection tube and the old tube and waste liquid discarded. Next, 500 ul AW1 buffer was added to each column before centrifugation for 1 minute at 8000 rpm. Again, the column was placed into a new collection tube, and the old tube and waste liquid discarded. Next, 500 uL of AW2 was added to each column before centrifugation for 3 minutes at 14000 rpm. Finally, the column was placed in a new 1.5 mL centrifuge tube and the old tube and waste liquid discarded. Following, 200 ul of elution buffer was added to the collection tube (onto the filter surface) and left for 1 minute at room temperature before a final centrifugation for 1 minute at 8000 rpm. Lastly, the column was disposed and centrifuge tube with DNA was labelled and stored at -20°C. Table 3.2 provides further detail regarding the specific function of solutions used in the DNA extraction process.

Table 3.2. Functions of solutions used in DNA extraction process.

Solution	**Function**
ATL Buffer, AL Buffer	Lysis solutions that open tissues and dissolve membrane bound organelles including the nucleus and mitochondria.
Proteinase K	Rapidly digests protein, including enzymes that digest DNA.
Ethanol	Allows for precipitation of DNA.
AW Buffers (1 & 2)	Wash solutions that wash away contaminants from DNA.
Elution Buffer	Elutes DNA from the membrane and allows stable storage of DNA.

DNA Confirmation and Quality Check

Presence of DNA in DNA extraction samples was confirmed by gel electrophoresis. A 1% gel was created using 30 mL of 0.5M Tris/Borate/EDTA (TBE) buffer solution and 1.2 g agarose (high gel strength, Sigma Aldrich). TBE and agarose were measured, combined and microwaved at high power until reaching a clear liquid phase. Following, 7.2 uL of ethidium bromide (EtBr) stain was added to the solution. The gel was then poured into a gel tray (approximately 15 cm x 12 cm, with well comb) and left to set for approximately 20-30 minutes.

While the gel was setting, DNA samples were removed from the freezer and allowed to thaw at room temperature. Following, 5 uL of DNA sample, 5 uL of molecular grade water (i.e. High Performance Liquid Chromatography grade water, henceforth referred to as HPLC-grade water) and 3 uL of loading dye were added to a separate 1.5 mL centrifuge tube for each sample. Additionally, 1 uL of DNA ladder marker (λ Hind III, Thermo Fisher Scientific) and 5 uL of HPLC-grade water were added to a separate centrifuge tube. All tubes were pulsed in the centrifuge.

Once set, the gel was placed into an electrophoresis tank and 0.5M TBE buffer was added until the gel was covered and all wells filled. 5 uL of the DNA ladder marker solution was added to the first well and 10uL of all DNA sample solutions to subsequent wells. The electrophoresis tank was then turned on (100-150V) and the gel left to run for approximately 30-45 minutes (until samples ran approximately ¾ gel length). The gel was then removed from tank and imaged under ultraviolet light to check presence and

quality of DNA. The presence of a distinct single band of high molecular weight indicated successful DNA extraction while a smear indicated poorer quality (Figure 3.2).

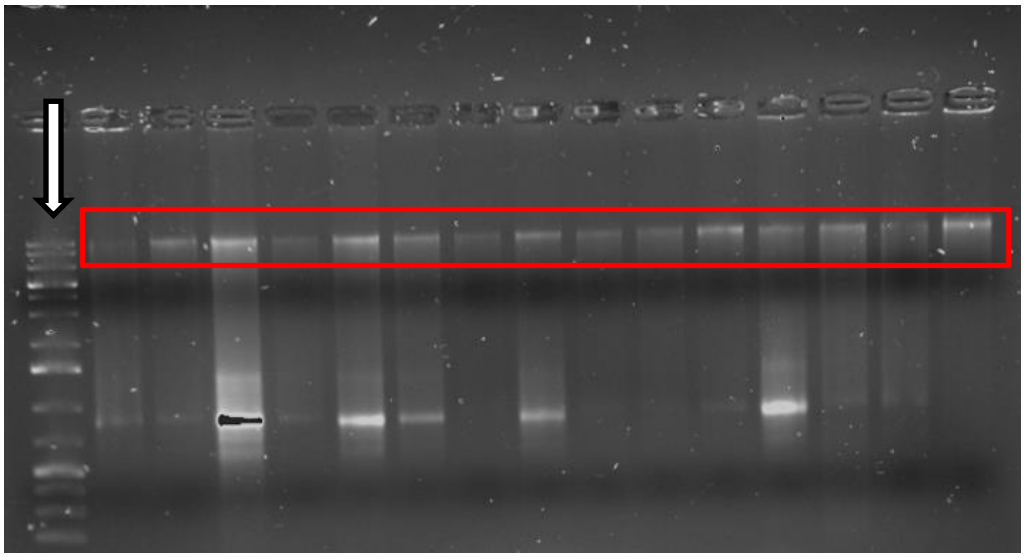


Figure 3.2. Electrophoresis gel confirmation of *M. modiolus* DNA extractions. The top bands (indicated by red box) show that a reasonable yield of high molecular weight DNA is present. The λHind III DNA sizing ladder (indicated by white arrow) shows that DNA extractions are approximately 23130 base pairs in size.

DNA Quantification

Following confirmation of DNA presence and quality, DNA was quantified for each sample. A biophotometer (Eppendorf, Stevenage, UK) programmed for double stranded DNA was used to quantify DNA samples. Samples were removed from the freezer and thawed at room temperature. 5 uL of elution buffer (as used for DNA extraction) and 45 uL of HPLC-grade water were added to a clean plastic cuvette and used as a blank. Following, the cuvette was emptied and rinsed with HPLC-grade water before a 5 uL sample of DNA extraction with 45 uL of HPLC-grade water was added and quantified. Readings were recorded in ng DNA uL^{-1}. The cuvette was rinsed with HPLC-grade water between measurements. Following quantification, samples were diluted to 20ng uL^{-1} with Tris-EDTA buffer solution (PH 8.0) for running in PCR.

Microsatellite Screening

M. modiolus microsatellite markers (named Modimicro 2, 11, 13, 20, and 30) previously developed by Gormley (2014) (described in Gormley *et al.*, 2015) were used to screen all populations (Table 3.3). Fluorescently labelled forward primers and non-labelled reverse primers for loci were ordered from Eurofins (FAM and HEX dyes) and Applied Biosystems (NED dye).

PCR Amplification

Polymerase chain reaction (PCR) with each of the five markers was carried out with all samples. PCR reactions were prepared using Illustra PureTaq Ready-To-Go PCR beads (GE Healthcare, Hatfield, UK) with 1 uL of DNA sample (diluted to 20 ng uL^{-1}), 1 uL of forward primer, 1 uL of reverse primer and 22 uL of HPLC-grade water for a final reaction volume of 25 uL. A negative control reaction was also set up using 1 uL of forward primer, 1 uL of reverse primer and 23 uL of HPLC to ensure no contamination of markers or HPLC-grade water. PCR reactions were run on a G-Storm (Kapa Biosystems, Wilmington, USA) thermocycler according to the following conditions:

- 94°C for 5 mins.
- 30 cycles of: 94°C for 30 s (denaturation); 53°C to 58.1°C for 30 s (annealing); 72°C for 30 s (elongation).
- Final step at 72°C for 5 mins.
- Held at 4°C until storage.

Table 3.3. *M. modiolus* microsatellite markers used in preliminary connectivity analyses. Forward and reverse sequences (5' – 3') and size data (bp) shown for Modiomicro 2, 11, 13, 20 and 30. Fluorescent label indicated for forward sequences.

Marker Name			Sequence (5' - 3')	Fluorescent Label	Amplicon size (bp)
Modimicro	2	F	CTCCGCTATGTTTGACCATGTA	FAM	500/300
Modimicro	2	R	TCCACACCGAGTAACAAATCAG	n/a	
Modimicro	11	F	AGAATCCTTTCTGTGTTGTCCG	HEX	400/100
Modimicro	11	R	CATCTGCCTACCTACAGTTCCC	n/a	
Modimicro	13	F	CACAGCCTCCTGGTCACAATA	HEX	300/100
Modimicro	13	R	TGGCGTGTTATTCTAGCAAATG	n/a	
Modimicro	20	F	AATTGCTCACTTGGCGTAAAAC	NED	200/300
Modimicro	20	R	TGGAAATGGAGAGACAGATCCT	n/a	
Modimicro	30	F	CACACAAGACAGGCCAGATAGA	FAM	700/200
Modimicro	30	R	GAAGAATCCCCACAAACACATT	n/a	

F=forward primer; R=reverse primer; bp=base pairs; n/a=not applicable

PCR amplification was confirmed with gel electrophoresis (Figure 3.3). Gels were created and run as described previously (refer to pp. 69-70) except for the following modifications: The ladder sample was prepared with 1 uL of 100 bp marker, 3 uL of loading dye and 4 uL of HPLC-grade water; and PCR samples were prepared with 5 uL of PCR product, 3 uL of loading dye, and 5 ul of HPLC-grade water. Following confirmation of successful amplification, PCR product was frozen (-20°C) until Fragment Size Analysis (FSA). 10 uL of PCR product per sample was transferred to a 96 well plate, sealed and sent on ice to University of Dundee genotyping service. If there was a delay before sending samples, PCR product was kept at -20°C until delivery.

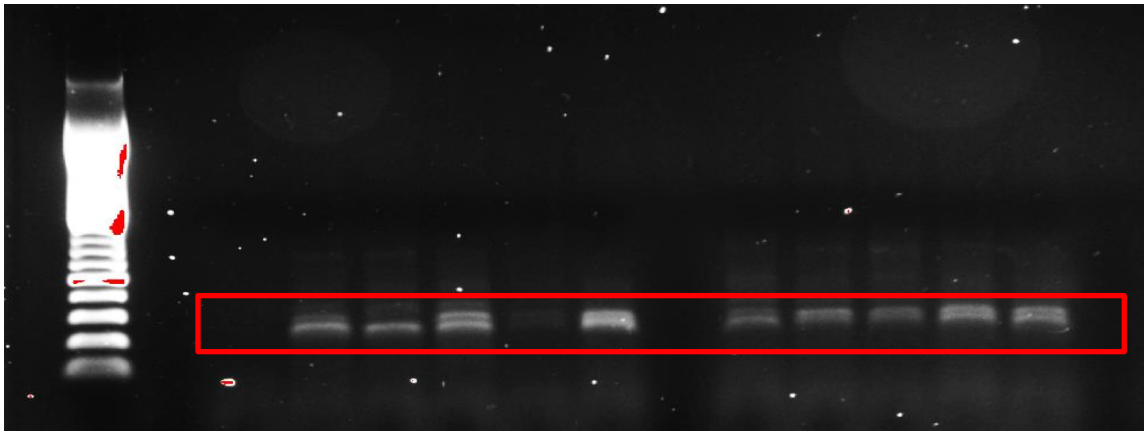


Figure 3.3. Gel electrophoresis confirmation of PCR amplification for Karlsruhe populations using Modimicro 20 microsatellite marker. Double bands at end of well lanes (indicated by red box) indicate successful amplification of target alleles.

Fragment Size Analysis

At University of Dundee, PCR fragments were run alongside a ROX500 size standard in an ABI 3130 Genetic Analyser. Alleles were scored using GeneMapper version 4.1 (Applied Biosystems, Foster City, USA). Scored peak graph images were received as Acrobat Adobe .pdf files (Figure 3.4) from University of Dundee. Peaks determined as representing the correct allele sizes were tabulated in Microsoft Excel software according to population and marker (Table 3.4).

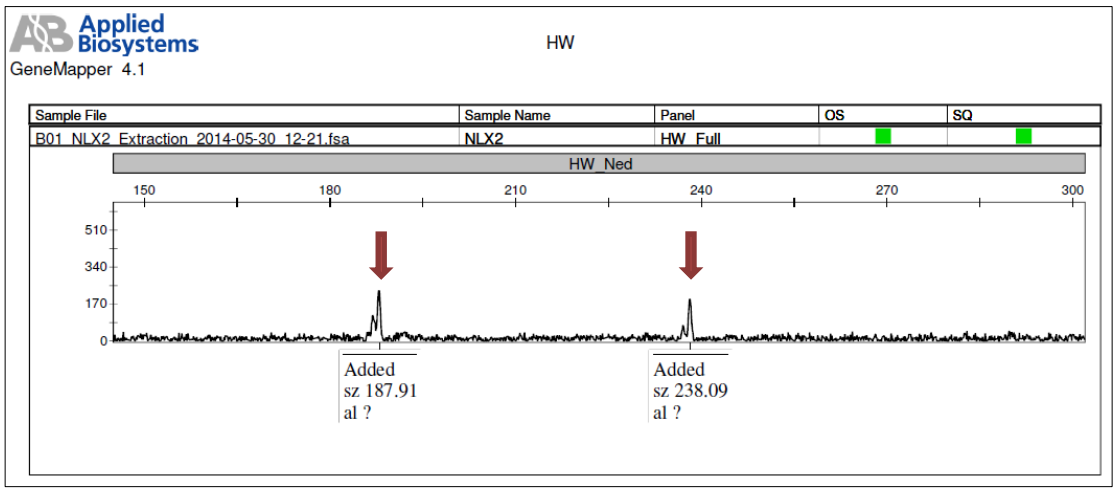


Figure 3.4. Example of allele peak data (allele peak scores indicated by red arrows) as received from University of Dundee. Example shows results from screening a North Lleyn sample with microsatellite marker Modimicro 20.

Table 3.4. Example of peak data for microsatellite markers Modimicro 2, 11, 13, 20, 30 for North Lleyn site (NLx) samples 1-5, as tabulated in MS Excel. Values represent the allele size as determined by peak values. Grey shading indicates no available data.

	2	2	11	11	13	13	20	20	30	30
NLx1	209	209	277	282	181	183	187	238	170	170
NLx2	219	254	282	285	182	182	188	238	172	172
NLx3	235	247	282	282	181	181	187	238	174	174
NLx4	209	233	282	282	181	181	187	238	171	171
NLx5			282	282	183	183			166	174

Data Analyses

An initial analysis was carried out by the author for North Lleyn, Point of Ayre, Strangford Lough, Port Appin and Karlsruhe populations to determine broad-scale patterns of genetic connectivity and diversity across the UK range of the habitat. Microsoft (MS) Excel software with the MS Tools Add-in was used to generate files for genetics analyses. Peak data output files were then applied to (1) FreeNA software (Chapuis & Estoup, 2007) to determine genetic differentiation (F_{st} of Weir (1996)), allelic frequency and richness, and presence of null alleles; (2) Fstat software (V2.9.3) (Goudet, 1995) to determine inbreeding coefficient (F_{is}), allelic frequency and number of alleles, and carry out pairwise significance tests of differentiation; and (3) Arlequin software v3.5

(Excoffier & Lischer, 2010) to determine deviations of genotype frequencies from the Hardy-Weinberg Equilibrium (HWE) via quantification of observed heterogeneity (Ho) and expected heterogeneity (He). Estimation of Null Allele (ENA) correction was performed (using FreeNA software) and pairwise F_{st} was calculated with and without ENA correction as described in Chapuis & Estoup (2007).

Following, a more focused analysis was completed in collaboration with Dr. Kate Gormley for Irish Sea populations (North Lleyn, Point of Ayre, Strangford Lough, Ards Peninsula) to examine connectivity in a regional sea and to determine transplant suitability between Northern Ireland populations (Ards Peninsula and Strangford Lough). Analyses was as described above (pp. 72-73).

Part 2: Application of Microsatellite Screening to Management: Considering the role of MPAs

Site Selection

Scotland

Three geographic regions in Scotland were selected to allow for an investigation into the relationship between *M. modiolus* reef populations at varying spatial scales and under varying oceanographic conditions (e.g. exposed coast vs enclosed sea lochs with restricted tidal exchange). Regions included the West Coast (Port Appin, Loch Creran and Oban Bay populations), North Coast and Orkney (Noss Head, Dornoch Firth and Karlsruhe populations) and Shetland Islands (Calback Ness, Uyea Sound and Hascosay Sound populations) (Figure 3.5). Within regions, *M. modiolus* reef sites ranged from as little as 10 km apart (e.g. Loch Creran to Port Appin) to distances >100 km (e.g. Karlsruhe to Dornoch Firth). Sites across the entire study area were as much as much as 700km apart (e.g. Loch Creran to Hascosay Sound). Each region also included at least one MPA site (Table 3.5). As the largest known *M. modiolus* reef in the UK (and highly abundant), the Noss Head population was also selected for additional examination of self-recruitment potential via examination of genetic differences between adults and juveniles.

Isle of Man

Three well-known IOM reef sites were selected to include in this investigation, including Point of Ayre, Ramsey Bay and Little Ness (Figure 3.6), as per the requirements of the IOM Government. The IOM sites included two designated sites (Ramsey Bay and Little Ness).

Animal Collection

Adult (>80mm shell length) *M. modiolus* (n=50) were hand collected by the Heriot-Watt Dive Team from all selected sites (except Ramsey Bay, sampled by IOM Government via grab sampler) between 2011-2016 (Table 3.5). Additionally, juveniles (<40mm shell length, as per Anwar *et al*., 1990) *M. modiolus* (n=50) were collected from Noss Head. All animals were transported in cold boxes (seawater and ice packs) to aquaria at Heriot-Watt University and kept in large flow through tanks (~15^0C) prior to processing.

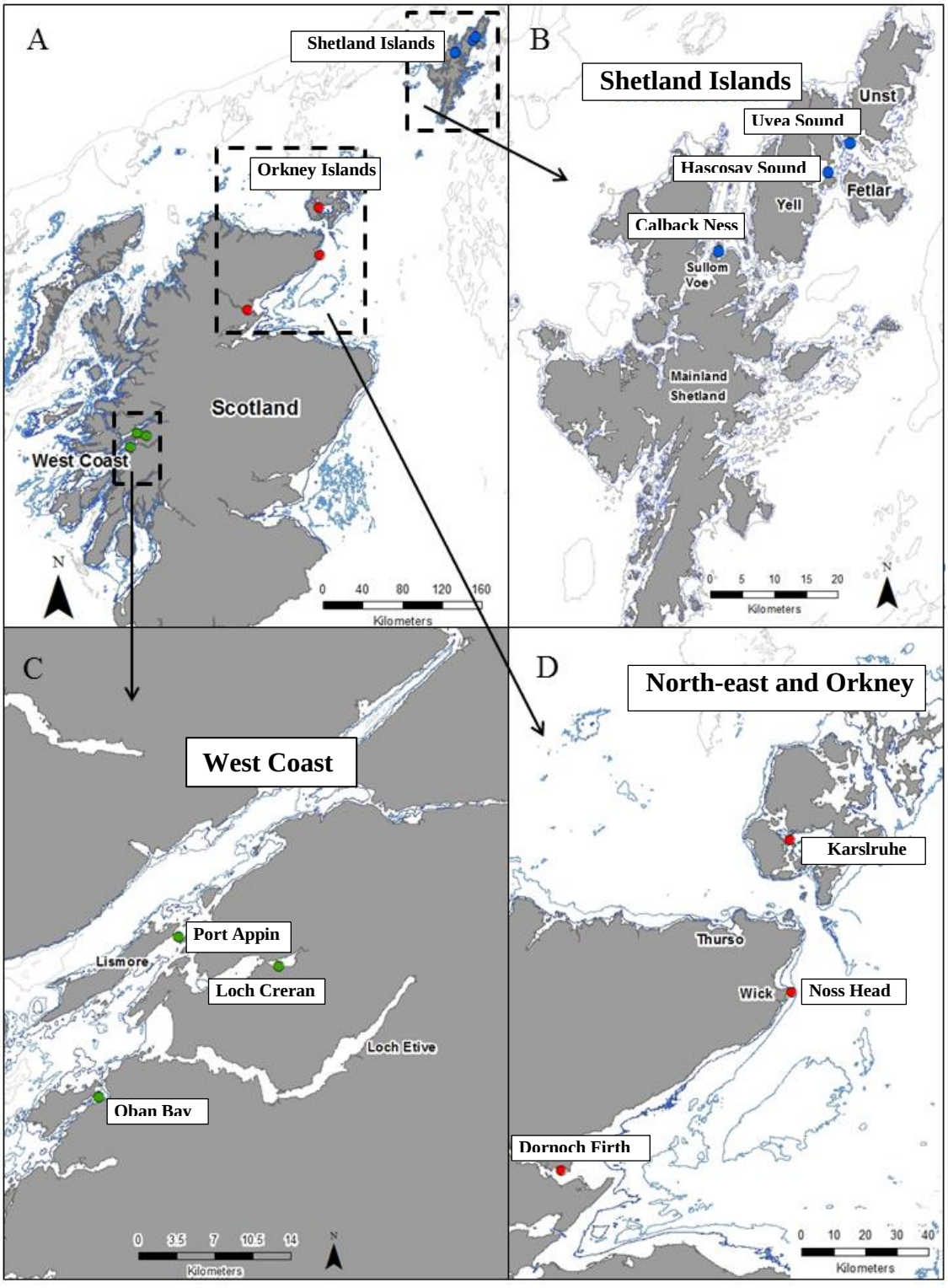


Figure 3.5. (A) *M. modiolus* reef populations included in genetic connectivity and diversity analyses of Scottish reef populations. Three regions (indicated by dashed boxes) were examined: (B) Shetland Islands, (C) West Coast and (D) North-east and Orkney. Reef sites are indicated by coloured markers and black bordered text.

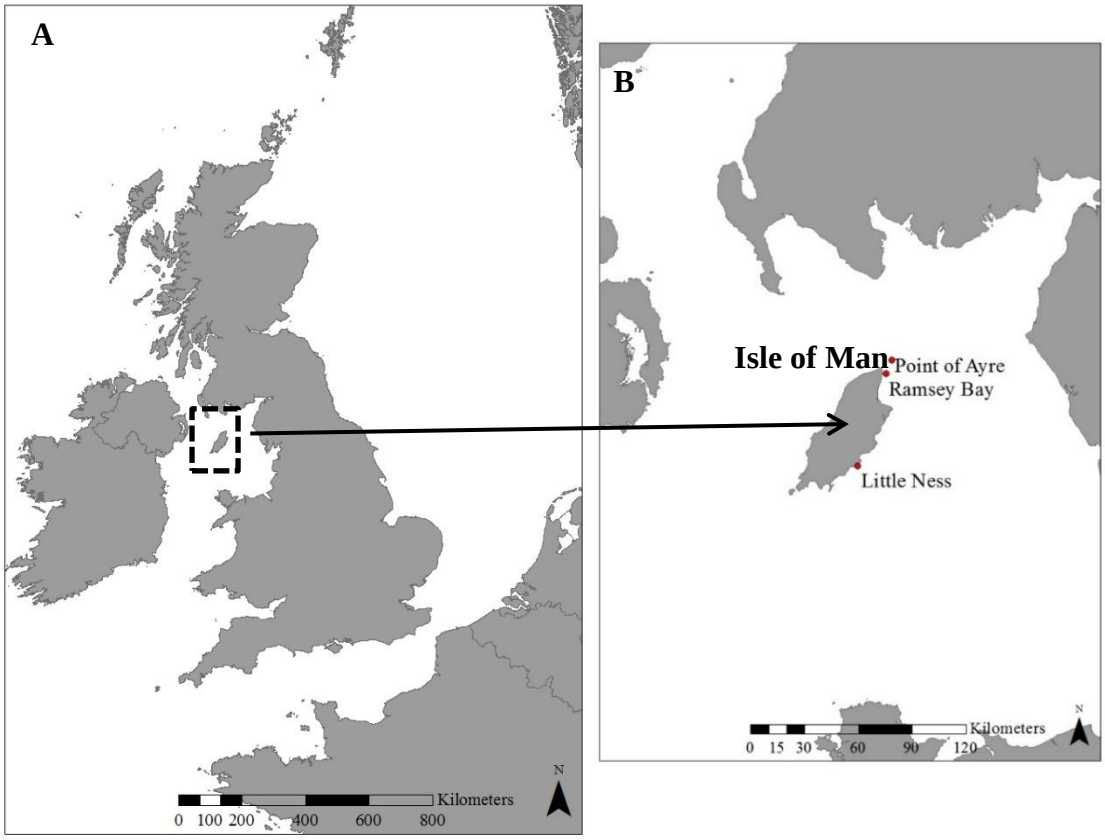


Figure 3.6. *M. modiolus* reef populations included in genetic connectivity and diversity analyses of Isle of Man reef populations: Point of Ayre, Ramsey Bay, Little Ness. Dashed box on left hand map (A) shows location of Isle of Man within the larger UK region. Reef locations indicated by red dots on right hand map (B).

Table 3.5. Collection data for *M. modiolus* reef sites included in genetic connectivity and diversity analyses of Scottish and IOM sites. Site name (and code), latitude and longitude, and collection date are provided for regions of Scotland (a-c) and Isle of Man (d). Additionally, designation (Designat.) type is indicated where horse mussel reefs are a protected feature.

Region	Site Name	Latitude	Longitude	Collection Date	Designat.
(a) **North-east and Orkney**	Dornoch Firth (DF)	57.857	-4.056	June 2016	SAC
	Noss Head (NH)*	58.470	-3.019	July 2016	MPA
	Karlsruhe (K)	58.889	-3.19	September 2011	*n/a*
(b) **West Coast**	Loch Creran (LC)	56.546	-5.269	January 2016	MPA, SAC
	Port Appin (PA)	56.551	-5.424	November 2011	*n/a*
	Oban Bay (OB)	56.412	-5.487	October 2011	*n/a*
(c) **Shetland Islands**	Hascosay Sound	60.618	-1.009	September 2012	MPA
	Uyea Sound (US)	60.667	-0.944	September 2012	MPA
	Calback Ness (CN)	60.483	-1.283	September 2012	SAC
(d) **Isle of Man**	Ramsey Bay (RB)	54.401	-4.344	September 2014	MPA
	Point of Ayre (POA)	54.439	-4.305	June 2013	*n/a*
	Little Ness (LN)	54.129	-4.475	June 2016	MPA

MPA=Marine Protected Area; SAC=Special Area of Conservation; *additional juveniles (n=50) collected from site.

Sample Preparation

Sample preparation was carried out as described in Part 1 (refer to p. 68)

DNA Extraction

DNA extractions were carried out as described in Part 1 (refer to pp. 68-69)

DNA Confirmation and Quality Check

DNA confirmation and quality checks were carried out as described in Part 1 (refer to pp. 69-70).

DNA Quantification

DNA quantification was carried out as described in Part 1 (refer to p. 70)

Microsatellite Screening

12 microsatellite markers were selected for screening of samples. Markers included four previously developed by Gormley (2014) (and described in Gormley *et al*., 2015) and 8 newly developed at the University of Manchester (Table 3.6). All primer sets were ordered from Eurofins Genomics (0.05 umol, salt-free, lyophilised) in February 2016. Upon receipt, primers were made to 100uM in Tris-EDTA buffer soluton (pH 8.0) and stored at -20°C until use.

Multiplex PCR

A multiplex PCR methodology was used to screen samples with the 12 selected microsatellite markers. This technique involves running PCR reactions that consist of multiple pairs of microsatellite markers per reaction. Locus-specific reverse (3'-5') primers and fluorescently labelled locus-specific forward (5'-3') primers for each marker set allow for multiple markers to be screened at one time. Multiplex Manager software (Holleley & Geerts, 2009) was used to design and optimise multiplex PCRs. Multiplex Manager combines markers to maximise spacing between markers in the same reaction and to minimise the variance of annealing temperature in each reaction. All twelve markers were inputted into the program and resulting multiplex groupings are shown in Table 3.7. For the current investigation, three multiplex PCR reactions were designed with four sets of microsatellite markers used per reaction. Forward markers were labelled with unique fluorescent dyes (NED, HEX, FAM, and PET dyes) to distinguish markers (and product) within a given set (Table 3.7).

Table 3.6. Microsatellite markers used in genetic analyses including forward and reverse primer sequences, amplicon size range, repeat motif, annealing temperature and marker developer.

Locus	**Forward Primer**	**Reverse Primer**	**Size range**	**Repeat motif**	**Annealing temp (°C)**	**Developed by**
MM13	CACAGCCTCCTGGTCACAATA	TGGCGTGTTATTCTAGCAAATG	150-200	GAA	57	Gormley (2014)
MM20	AATTGCTCACTTGGCGTAAAAC	TGGAAATGGAGAGACAGATCCT	180-248	TCA	57	Gormley (2014)
MM2	CTCCGCTATGTT TGACCATGTA	TCCACACCGAGTAACAAATCAG	116-317	CA	57	Gormley (2014)
MM30	CACACAAGACAGGCCAGATAGA	GAAGAATCCCCACAAACACATT	147-183	CA	57	Gormley (2014)
MM_pp19	GGTCGTTCCCTTTGACATGAACCC	AAACATCTTTCGCACCCGTTTGCC	384-389	AT	60	Manchester University*
MM_pp15	TGAGGTTAGTGAAAACTAATTGAGCAACCC	CGTTTCAGATTCTCCTTACAATTTGCC	357-369	ATT	60	Manchester University*
MM_pp27	TTTACTGAGTTCACACTGTTTTGCC	GCATCATATGTTACCCGTTCCC	310-326	AT	60	Manchester University*
MM_pp07	TCCAGGTATTTTAGTTCCAGAGATAGGG	GATTATTCATCTTGGAGCCATTGCC	304-308	CGG	60	Manchester University*
MM_pp17	TCTTACAGATTCGGGATTGTGAACCG	TCAACTTCAATCTTTTGGCCTTATCGG	235-260	AC	60	Manchester University*
MM_pp37	CCGTTGTGGATTTGTGAGAATACGC	GCGACTTAGTTCCACGCTTTTATTACGG	227-272	AT	60	Manchester University*
MM_pp24	TTTTCCTTTCTCTCTCCGCATTCGC	TGCTACCAAGGTTGTAACGAGATTCCC	292-309	AT	60	Manchester University*
MM_pp05	ACACCAAATTTAGCCCCTTTAGGC	AAAACCAAATGTTCCACCTAACCC	266-292	TCG	60	Manchester University*

*Preziosi & Rowntree Labs, Faculty of Life Sciences, Manchester University

Table 3.7. Results of Multiplex Manager for design and optimisation of multiplex PCR reactions including multiplex group (1-3), marker name, and labelling dye (for forward primer only).

Multiplex Group	Marker	Dye*
1	MM13	FAM
1	MM20	VIC
1	MM2	NED
1	MM30	PET
2	MM_pp19	FAM
2	MM_pp15	VIC
2	MM_pp27	NED
2	MM_pp07	PET
3	MM_pp17	FAM
3	MM_pp37	VIC
3	MM_pp24	NED
3	MM_pp05	PET

*attached to forward primer

Prior to running PCR, a primer mix was created for each multiplex PCR reaction (1-3) per amounts shown in Table 3.8. Primer mixes were created in 0.2 mL capped tubed and stored in a light-proof box at 4°C until use. Multiple aliquots were created to avoid cross contamination.

Prior to screening large numbers of samples, all multiplex PCR reactions (1-3) were tested across a small number of samples (n=5) from multiple populations (n=3) to validate the protocol. Multiplex PCR reactions were carried out using Qiagen Type-it Microsatellite PCR Kit (Qiagen, Manchester, UK) and protocol but prepared to a final reaction volume of 5µl (to maximise consumables), comprising:

- 2.5 µL TypeIT MasterMix
- 1.5 µL H_2O (molecular grade)
- 0.5 µL primer mix (as prepared above)
- 0.5 uL normalised DNA template (diluted to 20 ng/uL)

Multiplex PCRs were run on a G-Storm (Kapa Biosystems, Wilmington, USA) thermocycler according to the following conditions:

- 95°C for 5 mins.
- 35 cycles of: 95°C for 30 s; 60°C for 90 seconds; 72°C for 30s.
- Final step at 60°C for 30 mins.
- Held at 4°C until storage.

Table 3.8. Primer mixes prepared for multiplex PCR reactions (1-3) each consisting of four fluorescently labelled forward markers (denoted with _F), four reverse primers (denoted with _R) and made up to 50 uL volume with molecular grade water.

MULTIPLEX PCR REACTIONS (1-3) *(Markers listed vertically)*			**Volume** **(per multiplex)**
Multiplex 1	***Multiplex 2***	***Multiplex 3***	
MM13_F	MM_pp19_F	MM_pp17_F	1.0 uL
MM20_F	MM_pp15_F	MM_pp37_F	1.0 uL
MM2_F	MM_pp27_F	MM_pp24_F	1.0 uL
MM30_F	MM_pp07_F	MM_pp05_F	1.0 uL
MM13_R	MM_pp19_R	MM_pp17_R	1.0 uL
MM20_R	MM_pp15_R	MM_pp37_R	1.0 uL
MM2_R	MM_pp27_R	MM_pp24_R	1.0 uL
MM30_R	MM_pp07_R	MM_pp05_R	1.0 uL
		Molecular grade (HPLC) water	42 uL
		Total reaction volume	50 uL

PCR of initial test samples was followed by gel electrophoresis to confirm correctly sized bands (Figure 3.7). Following confirmation of successful trial multiplexes, multiplex PCRs were carried out as described above for all remaining samples (13 populations x 50 samples). A sub-sample (~10%) of each set of PCR samples was run on PCR gel to confirm successful amplification. Following gel confirmation of successful PCR, all product was transferred to a 96-well plate (10 ul per sample), sealed and sent on ice to University of Dundee for genotyping. If there was a delay before sending samples, PCR product was kept at -20°C until delivery. PCR was carried out with 50 samples at a time and 150 samples were sent in one batch for genotyping at a given time.

At University of Dundee, PCR fragments were run alongside a ROX500 size standard in an ABI 3130 Genetic Analyser. Output files were received in FAS file format and Peak Scanner (version 2) (Applied Biosystems, 2006) software was used to score peaks (Figure 3.8). Peaks scores represented the number of base pairs per allele (i.e. length of microsatellite region). All peak scores were entered in MS Excel software for subsequent analyses.

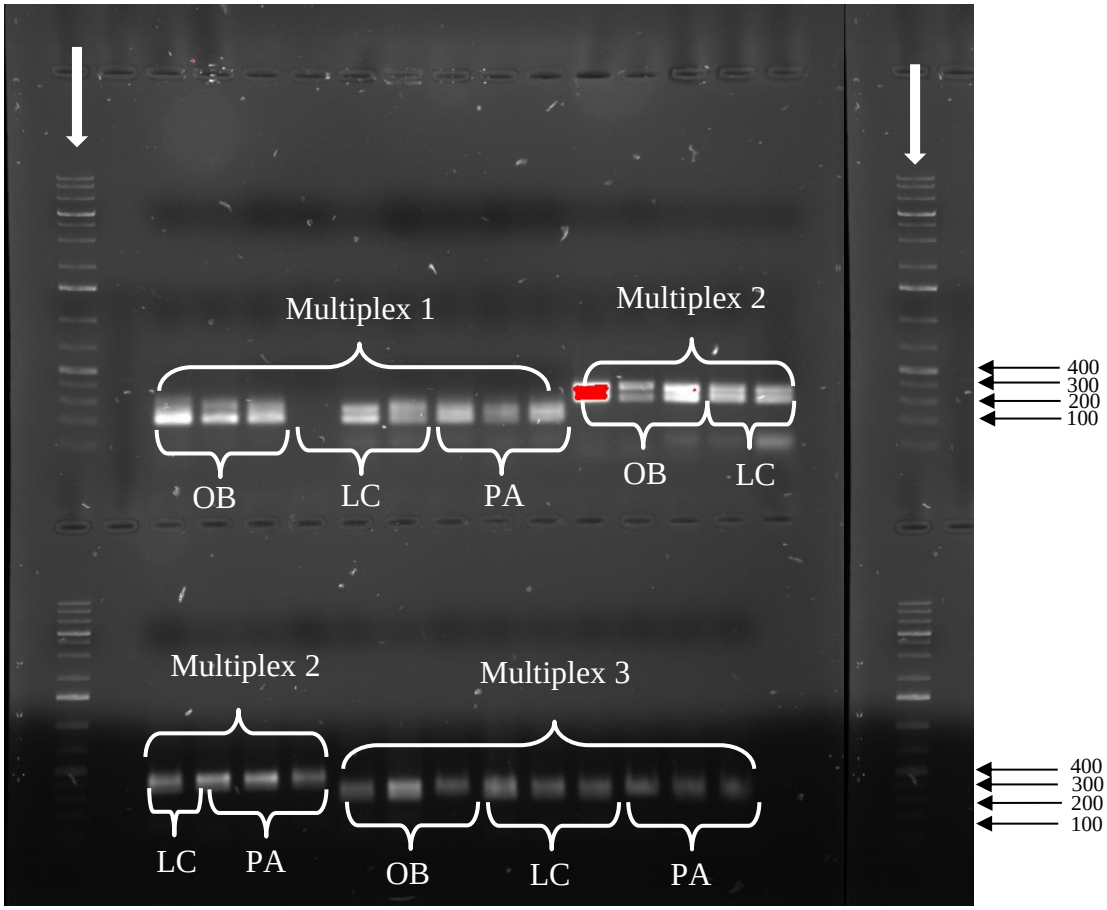


Figure 3.7. Gel electrophoresis results of trial multiplex (1-3) PCR of DNA samples (n=3) from three populations (OB: Oban; LC: Loch Creran; PA: Port Appin). 100 kb ladders (indicated by white arrows) to sides shows approximate size (bp) of amplicons.

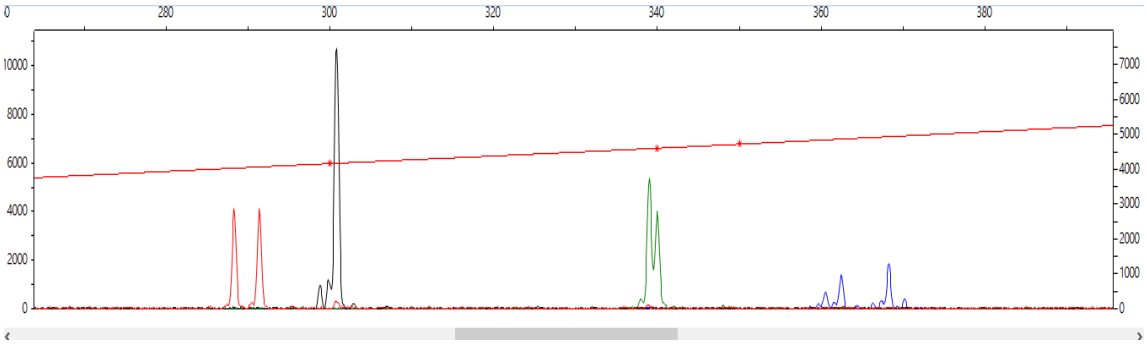


Figure 3.8. Example of peak data as visualised in Peak Scanner software. Different coloured peaks indicate different microsatellite markers. The position of the peak along the x-axis indicates the size of the particular allele. The height of a peak is less important.

Data Analyses

Initial data analyses for determination of diversity metrics and differentiation scores were carried out as outlined in Part 1 (refer to pp. 73-74). Additionally, population structure was inferred using STRUCTURE software (version 2.3.1) (Pritchard *et al.*, 2000; Falush *et al.*, 2003; 2007). STRUCTURE assigns individuals to populations (i.e. groups, denoted *K*) and identifies distinct genetic populations. STRUCTURE parameters were set to allow for admixture and correlated allele frequencies with a burn-in period of 100,000 followed by 500,000 iterations. Outputs of five iterations of *K*=1-8 were run in STRUCTURE Harvester web v0.6.92 (Earl & vonHoldt, 2012) to determine the uppermost true (estimated) number of genetic units (*K*). Evaluation of the Delta*K* and L(*K)* plots with the Evanno Method (Evanno *et al.*, 2005) identified the uppermost *K* value.

Migration-N software (version 3.6) (Beerli & Palczewski, 2010) was used to determine migration rates (*m*) of populations. To clarify, *m* is the fraction of immigrants in a population coming from another population over the most recent generation (i.e. immigrants per generation) (Figure 3.9) and therefore sometimes referred to as a backward migration rate (Beerli & Palczewski, 2010). MSAnalyser software (Dieringer & Schlotterer, 2003) was used to generate the Migrate-N input file. *m* values were inferred via Bayesian inference according to Fst values and using the Brownian motion model for microsatellites. A burn-in period of 1000000 was followed by 500000

iterations with constant mutation rates among loci assumed. Migration rates were compared between regions and within regions.

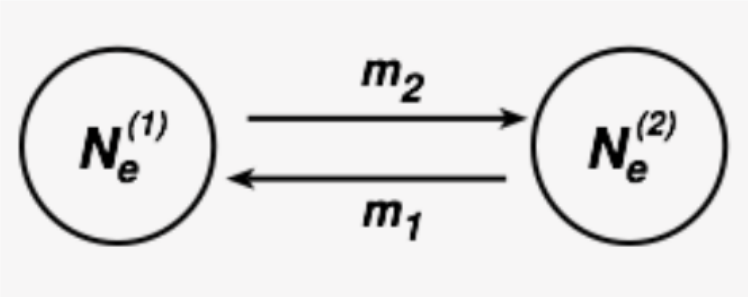


Figure 3.9. Migration rates (*m*) refer to the number of immigrants arriving over the last generation. For example, in the diagram above, m_1 refers to individuals moving from $N_e^{(2)}$ to $N_e^{(1)}$ while m_2 refers to individuals moving from to $N_e^{(1)}$ to $N_e^{(2)}$ (Image source: Beerli & Palczewski, 2010).

3.5 Results

Part 1: Preliminary Microsatellite Screening of UK Populations

Preliminary analyses indicated that Strangford Lough population had the strongest levels of differentiation from other populations. Strangford Lough was significantly moderately[3] differentiated from North Lleyn, Point of Ayre and Port Appin populations. Results also showed that the most northern population (Karlsruhe) had significantly low levels of differentiation with the three most southern population populations (Strangford Lough, Point of Ayre and North Lleyn) (Tables 3.9 & 3.10). Likewise, results indicated that the Port Appin population had significantly low levels of differentiation with both North Lleyn and Point of Ayre populations. Null alleles were detected across all markers and populations ($r > 0.05$).

[3] For the interpretation of Fst, a value lying in the range 0–0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (Wright, 1978; Hartl & Clark, 1997).

Local inbreeding coefficients (F_{is} scores) of all populations ranged from 0.047 (Port Appin) - 0.400 (North Lleyn) (Table 3.9). Significant positive F_{is} values[4] for all populations indicated a deficiency of heterozygotes compared to that expected under the Hardy-Weinberg Equilibrium (HWE). Therefore, it was concluded that all populations were not in HWE. All populations also had lower H_o values (ranging from 0.4322-0.6361) than H_e values (ranging from 0.6241-0.8450), further indicating a heterozygote deficiency across all populations (Table 3.9). Strangford Lough and North Lleyn showed the largest deficit in H_e compared to H_o, indicated larger deviation from HWE, while Point of Ayre and Port Appin showed the smaller deficit in H_e from H_o, indicated lesser deviation from HWE. Strangford Lough and Point of Ayre had the highest and lowest number of alleles and allelic richness, respectively (Table 3.9).

Follow-up analyses focusing solely on Irish Sea populations showed low but significant differentiation of Northern Ireland *M. modiolus* populations from Irish Sea counterparts (Ards Peninsula-Point of Ayre: F_{st}=0.07; Ards Peninsula-North Lleyn: F_{st}=0.05; Strangford Lough-Point of Ayre: F_{st}=0.10; Strangford Lough-North Lleyn: F_{st}=0.07). Pairwise comparisons of the Strangford Lough and Ards Peninsula populations, and North Lleyn and Point of Ayre populations showed low but insignificant levels of differentiation (Tables 3.11 & 3.12).

[4] For interpretation of F_{is}: F_{is}= 0 indicates no inbreeding; F_{is}>0 indicates a heterozygote deficit (inbreeding); F_{is} <0 indicates a heterozygote excess.

Table 3.9. Diversity indices and differentiation values for UK *M. modiolus* populations (North Lleyn, Point of Ayre, Strangford Lough, Port Appin, Karlsruhe). Bold font F_{st} values indicate significant differentiation.

Diversity Indices							**F_{st} (before and after ENA correction)**				
Site	**N**	**H_e**	**H_o**	**N_A**	**A_R**	**F_{is}**	**North Lleyn**	**Point of Ayre**	**Strangford Lough**	**Port Appin**	**Karlsruhe**
North Lleyn	48	0.7171	0.4322	13.0	10.14 ± 6.96	0.400		0.0257	**0.0659**	**0.0270**	**0.0188**
Point of Ayre	31	0.6241	0.5819	9.4	8.84 ±5.88	0.069	0.0511		**0.1079**	**0.0141**	**0.0381**
Strangford Lough	51	0.8450	0.5958	20.8	14.65 ±8.43	0.297	**0.0645**	**0.1191**		**0.0893**	**0.0247**
Port Appin	41	0.6674	0.6361	14.2	10.85 ±9.58	0.047	**0.0471**	**0.0108**	**0.0986**		0.0345
Karlsruhe	54	0.7795	0.5769	15.8	11.75 ±5.90	0.262	**0.0225**	**0.0481**	**0.0240**	0.0432	

N = number of samples; H_e = Expected Heterozygosity; H_o = Observed Heterozygosity; N_A = number of alleles; A_R = Allelic Richness; F_{is} = inbreeding coefficient; F_{st} before (upper diagonal) and after (lower diagonal) ENA correction; *significance ($p<0.05$)

Table 3.10. Level (low, moderate, high) and significance of genetic differentiation values for UK *M. modiolus* populations (North Lleyn, Point of Ayre, Strangford Lough, Port Appin, Karlsruhe).

Population	North Lleyn	Point of Ayre	Strangford Lough	Port Appin	Karlsruhe
North Lleyn		ns	*	*	*
Point of Ayre			*	*	*
Strangford Lough				*	*
Port Appin					ns
Karlsruhe					
Level of Differentiation	LOW		MODERATE	HIGH	

*=significant differentiation; ns=non-significant differentiation

Table 3.11. Diversity indices and differentiation values for Irish Sea *M. modiolus* populations (North Lleyn, Point of Ayre, Strangford Lough, Port Appin, Karlsruhe). Bold font F_{st} values indicate significant differentiation.

Diversity Indices							**F_{st} (before and after ENA correction)**			
Site	**N**	**H_e**	**H_o**	**N_A**	**A_R**	**F_{is}**	**Ards Peninsula**	**Strangford Lough**	**North Lleyn**	**Point of Ayre**
Ards Peninsula	50	0.8178	0.5432	18.20	13.23 ±7.86	0.338		0.00	**0.05**	**0.07**
Strangford Lough	51	0.8450	0.5958	20.80	14.65 ±7.54	0.297	0.01		**0.07**	**0.10**
North Lleyn	48	0.7186	0.4373	13.00	10.14 ±6.23	0.394	**0.05**	**0.06**		0.02
Point of Ayre	31	0.6241	0.5819	9.40	8.84 ±5.26	0.069	**0.09**	**0.12**	0.05	

N = number of samples; H_e = Expected Heterozygosity; H_o = Observed Heterozygosity; N_A = number of alleles; A_R = Allelic Richness; F_{is} = inbreeding coefficient; F_{st} before (upper diagonal) and after (lower diagonal) ENA correction; *significance ($p<0.05$)

Table 3.12. Level (low, moderate, high) and significance of genetic differentiation values Irish Sea *M. modiolus* populations (North Lleyn, Point of Ayre, Strangford Lough, Port Appin, Karlsruhe).

Population	Ards Peninsula	Strangford Lough	North Lleyn	Point of Ayre
Ards Peninsula		ns	*	*
Strangford Lough			*	*
North Lleyn				ns
Point of Ayre				
Level of Differentiation	LOW		MODERATE	HIGH

Part 2: Application of Microsatellite Screening to Management: Considering the role of MPAs

Scotland

Results indicated minimal-moderate differentiation across all Scottish *M. modiolus* reef sites. However, comparison of regions suggested increased levels of differentiation within populations of the West Coast and North-east and Orkney regions as compared to those of Shetland Islands region. Within the West Coast region, Port Appin showed significant moderate differentiation from both Loch Creran (F_{st}=0.069) and Oban (F_{st}=0.081) populations while Loch Crean and Oban populations showed low differentiation (F_{st}=0.001). Within the North-east and Orkney region, Karlsruhe showed significant moderate differentiation from all other sites (Noss Head: F_{st}=0.1064 (adults), F_{st}=0.066 (juveniles); Dornoch Firth: F_{st}=0.090). Within Shetlands Islands region, all populations showed low differentiation but results were non-significant (Tables 3.13 & 3.14).

Across all populations, irrespective of region, several key patterns were detected. Firstly, Port Appin and Karlsruhe populations showed significant moderate differentiation from all other populations except Dornoch Firth where moderate differentiation values were deemed non-significant (as for all pairwise comparisons with Dornoch Firth). Additionally, West Coast populations were generally moderately differentiated from North-east and Orkney populations while only minimally differentiated from Shetland Islands populations. In contrast, results suggest that North-east and Orkney populations are minimally differentiated from Shetland Islands populations though results were insignificant. Conversely, the Orkney population (Karlsruhe) was found to be significantly moderately differentiated from all Shetland Islands populations (Tables 3.13 & 3.14).

Local inbreeding coefficients (F_{is} scores) of Scottish populations ranged from 0.176 (Uyea Sound) to 0.519 (Noss Head - adults), indicating a heterozygote deficit across all populations (Table 3.13). Likewise, all H_o values (ranging from 0.29373 in Noss Head adults to 0.56460 in Uyea Sound) were lower than H_e values (ranging from 0.6054 in Noss Head adults to 0.7203 in Noss Head juveniles), further indicating a heterozygote deficiency (Table 3.13). Therefore, it was concluded that all populations were not in HWE. Null alleles were detected across all markers and populations ($r > 0.05$).

Across all populations, Uyea Sound had the greatest number of alleles, highest H_o, lowest F_{is}, and a high allelic richness score (only lower than one other population), potentially suggesting increased genetic diversity in this population. Conversely, Noss Head (adults) had the lowest H_o and highest F_{is}. Regionally, the Shetland Islands appeared to have the greatest genetic diversity while genetic diversity seemed generally comparable between the other regions. Comparison of divergence between H_o and H_e values across all populations showed greatest discrepancies in Noss Head adults and Noss Head juveniles.

Table 3.13. Diversity indices and differentiation values for Scottish *M. modiolus* reefs. Regional groupings are indicated by coloured shading (blue=West Coast; red=North Coast and Orkney; orange=Shetland Islands). Bold font F_{st} values indicate significant differentiation.

	Diversity Indices						**Differentiation (F_{st} before and after ENA correction)**									
Population	N	H_e	H_o	N_A	A_R	F_{is}	Oban	Loch Creran	Port Appin	Karlsruhe	Noss Head (A)	Noss Head (J)	Dornoch Firth	Calback Ness	Uyea Sound	Hascosay Sound
Oban	49	0.6406	0.35658	8.92	48.62 (±1.20)	0.447		0.004	**0.086**	**0.108**	**0.053**	**0.062**	0.040	0.015	0.029	0.013
Loch Creran	49	0.6413	0.3147	8.83	47.80 (±1.09)	0.518	0.001		**0.070**	**0.104**	**0.056**	**0.062**	0.038	0.016	0.028	0.013
Port Appin	41	0.6922	0.42998	10.58	53.37 (±1.79)	0.382	**0.081**	**0.069**		**0.059**	**0.142**	**0.097**	**0.138**	**0.081**	**0.080**	**0.077**
Karlsruhe	49	0.6672	0.4423	11.08	53.26 (±1.73)	0.340	**0.095**	**0.091**	**0.056**		**0.116**	**0.070**	**0.106**	**0.079**	**0.054**	**0.083**
Noss Head (A)	50	0.6054	0.29373	8.75	50.15 (±1.81)	0.519	0.044	**0.051**	**0.137**	**0.1064**		0.037	0.022	0.036	0.040	0.029
Noss Head (J)	50	0.7203	0.40919	8.83	55.77 (±1.24)	0.436	0.043	0.049	**0.085**	**0.066**	0.035		0.031	0.031	0.032	0.033
Dornoch Firth	50	0.6262	0.33113	7.92	48.67 (±1.58)	0.477	0.024	0.023	**0.119**	**0.090**	0.020	0.026		0.029	0.028	0.027
Calback Ness	45	0.6926	0.5254	10.58	55.81 (±1.48)	0.244	0.010	0.014	**0.079**	**0.070**	0.032	0.027	0.019		0.010	0.002
Uyea Sound	50	0.6839	0.56460	12.83	55.11 (±1.48)	0.176	0.020	0.022	**0.084**	**0.0539**	0.034	0.034	0.019	0.009		0.013
Hascosay Sound	50	0.6888	0.49496	11.75	55.05 (±1.69)	0.284	0.011	0.013	**0.080**	**0.0775**	0.025	0.033	0.018	0.003	0.012	

N = number of samples; H_e = Expected Heterozygosity; H_o = Observed Heterozygosity; N_A = number of alleles; A_R = Allelic Richness; F_{is} = inbreeding coefficient; F_{st} before (upper diagonal) and after (lower diagonal) ENA correction; *significance ($p<0.05$)

Table 3.14. Level (low, moderate, high) and significance of genetic differentiation values for Scottish *M. modiolus* reefs.

Population	Oban	Loch Creran	Port Appin	Karlsruhe	Noss Head (A)	Noss Head (J)	Dornoch Firth	Calback Ness	Uyea Sound	Hascosay Sound
Oban		ns	*	*	*	ns	ns	*	*	*
Loch Creran			ns	*	ns	ns	ns	*	*	NS
Port Appin				*	*	*	ns	*	*	*
Karlsruhe					*	*	ns	*	*	*
Noss Head (A)						ns	ns	*	*	*
Noss Head (J)							ns	ns	ns	ns
Dornoch Firth								ns	ns	ns
Calback Ness									*	ns
Uyea Sound										ns
Hascosay Sound										
Level of Differentiation	LOW			MODERATE			HIGH			

*=significant differentiation; ns=non-significant differentiation

IOM

Results indicated significant moderate genetic differentiation between all IOM populations. Strongest differentiation was indicated between Little Ness and Point of Ayre populations and weakest differentiation between Ramsey Bay and Little Ness populations (Tables 3.15 & 3.16). F_{is} scores of IOM populations ranged from 0.136-0.256, indicating a heterozygote deficiency across all populations (Table 3.15). Likewise, all IOM populations had lower H_o values (ranging from 0.44644-0.60344) than H_e values (ranging from 0.59845-0.73246), further indicating a heterozygote deficiency. Therefore, it was concluded that all populations were not in HWE. Ramsey Bay had the highest H_o and H_e scores, number of alleles and allelic richness (Tables 3.15), suggesting greater genetic diversity than Point of Ayre and Little Ness populations. Null alleles were detected across all markers and populations ($r > 0.05$).

Table 3.15. Diversity indices and differentiation values for IOM (Little Ness, Point of Ayre and Ramsey Bay) populations. Bold font F_{st} values indicate significant differentiation.

Diversity Indices							**Differentiation (F_{st} before and after ENA correction)**		
Population	N	H_e	H_o	N_A	A_R	F_{is}	Little Ness	Point of Ayre	Ramsey Bay
Little Ness	50	0.59845	0.44644	107	47.2	0.256		**0.1138**	**0.0979**
Point of Ayre	31	0.61503	0.53308	98	49.0	0.136	**0.1082**		**0.0942**
Ramsey Bay	39	0.73246	0.60344	151	58.2	0.178	**0.0931**	**0.0989**	

N = number of samples; H_e = Expected Heterozygosity; H_o = Observed Heterozygosity; N_A = number of alleles; A_R = Allelic Richness; F_{is} = inbreeding coefficient; F_{st} before (upper diagonal) and after (lower diagonal) ENA correction (ENA = exclusion of null alleles).

Table 3.16. Level (low, moderate, high) and significance of genetic differentiation values for IOM (Little Ness, Point of Ayre and Ramsey Bay) populations.

Population	Little Ness	Point of Ayre	Ramsey Bay
Little Ness		*	*
Point of Ayre			*
Ramsey Bay			
Level of Differentiation	LOW	MODERATE	HIGH

*=significant differentiation; ns=non-significant differentiation

Structure Analyses and Migration Rates (Scottish and IOM populations)

STRUCTURE and STRUCTURE Harvester results found *K*=2 to be the most likely *K* value, indicating there are two main genetic groups across all Scottish and Isle of Man populations (Figures 3.10 & 3.11). Group 1 included populations of the West Coast: Loch Creran, Oban; North Coast: Noss Head (adults), Dornoch Firth; Shetland Islands: Hascosay Sound, Uyea Sound, Calback Ness; and Isle of Man: Little Ness. Group 2 included populations of the West Coast: Port Appin; Orkney: Karlsruhe; and Isle of Man: Point of Ayre and Ramsey Bay. Noss Head (juveniles) appeared to be part of both groups (though associated more strongly with Group 1) and thus potentially have a distinct structure as compared to Noss Head adults. STRUCTURE results for *K*=3 suggested a potential sub-group within Ramsey Bay and Noss Head (juveniles) and *K*=4 suggested a potential sub-group within Port Appin (Figure 3.10).

Migration rates for all populations (across all regions) are presented in Appendix B, Table B1. Migrate-N analyses determined average migration rates (i.e. number of immigrants generation^{-1}) of 12.7 (SD±4.29) for the West Coast region; 14.4 (SD±7.52) for the North-east and Orkney region; 14.3 (SD±4.41) for the Shetland Islands region; and 12.9 (SD±4.25) for the Isle of Man region. Migration rates ranged from 4.30-25.25 for the West Coast region; 4.41-54.34 for the North-east and Orkney region; 5.56-23.55 for Shetland Islands region; and 4.50-21.78 for the Isle of Man region. The strongest and weakest migration rates were from Loch Creran to Dornoch Firth (54.3) and Point of Ayre to Loch Creran (4.3), respectively.

A comparison of migration rates for populations within each region indicated directional bias (i.e. stronger unidirectional immigration from one population to another) to Port Appin and Oban in West Coast populations; to Karlsruhe, Noss Head (adults) and Dornoch Firth in North-east and Orkney populations; to Uyea Sound and Hascosay Sound in Shetland Islands populations; and to Point of Ayre and Little Ness in Isle of Man poulations (Table 3.17, Figure 3.12). However, in many cases, migration rates were quite comparable so directional bias may be negligible. Likewise, when mean migration rates from all other populations were calculated per population (Table 3.18), values and variation were generally comparable across sites. Immigration rates were highest for Dornoch Firth followed by Uyea Sound, and lowest for Noss Head (juveniles) followed by Loch Creran. Comparison of migration rates of Noss Head adults and juveniles

indicated a stronger migration from juveniles towards adults (22.7) than adult to juveniles (5.6).

Comparison of mean and sum migration rates (number of immigrants generation^{-1}) between Scottish regions (Table 3.19), showed directional bias from West Coast to North-east and Orkney, from Shetland Islands to West Coast and from Shetland Islands to North-east and Orkney. Additionally, results indicate higher levels of self-recruitment within North-east and Orkney region as compared to the other three regions. For demonstration, Figure 3.12 provides an overview of directional bias of migration within and across Scottish regions. All three Scottish regions show directional bias towards the Isle of Man region (Table 3.19)

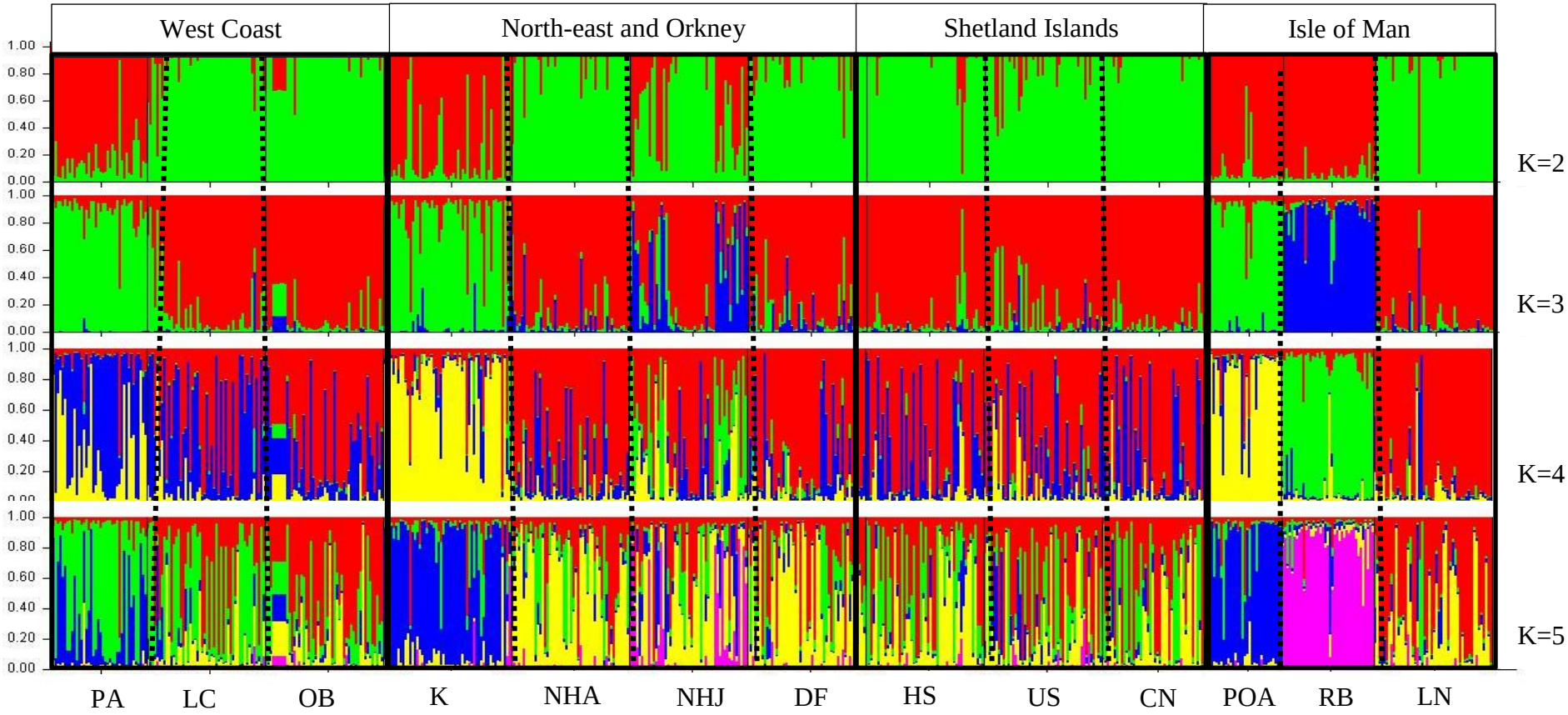


Figure 3.10. STRUCTURE output with K=2-5 for *M. modiolus* reef populations across Scottish regions (West Coast, North-east and Orkney, Shetland Islands) and Isle of Man. Population codes provide along x-axis (as per Table 3.5). Each bar represents an individual within a population. Populations showing the same colour pattern are part of the same genetic group (K). As K is increased, structure becomes less clear but highlights potential genetic relationships across all populations including a number of potential sub-groups (e.g. POA, RB, K).

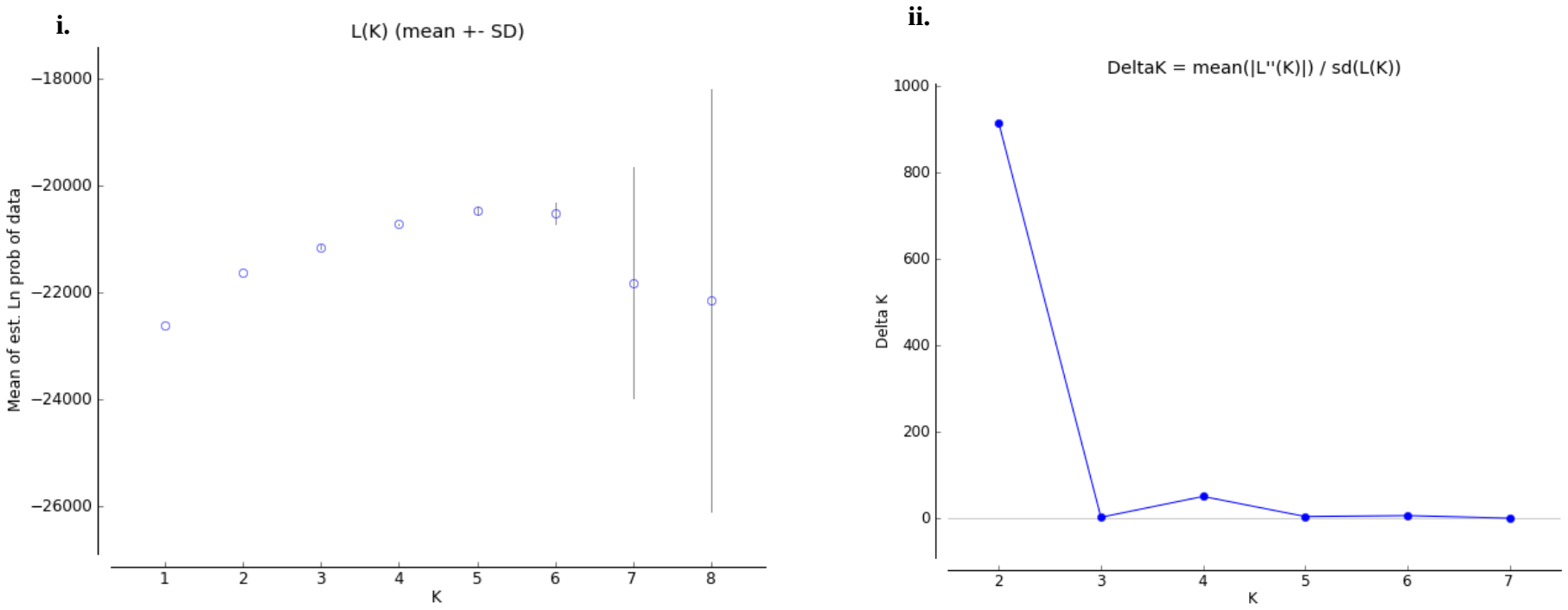


Figure 3.11. STRUCTURE Harvester (i) L(K) and (ii) DeltaK plots for determining best K value (plots generated by STRUCTURE Harvester).

Table 3.17. Pairwise estimates of migration (number of immigrants generation^{-1} into receiving population) between *M. modiolus* reef populations within Scottish regions (West Coast, North Coast and Orkney, Shetland Islands) and Isle of Man, as determined by Migrate-N software. Directional bias determined as the migration direction which resulted in a greater migration rate, though in most cases the difference is negligible. Full data of all comparisons available (refer to Appendix B, Table B1).

		Number of immigrants generation^{-1} into receiving population			
Region	**Migration direction (sink-source)**	**2.5% percentile**	**Mean**	**97.5% percentile**	**Directional bias**
West Coast	mLC-AP	6.7	13.8	30.0	LC to AP
	mAP-LC	4.0	11.3	28.0	
	mOB-AP	4.0	11.0	27.3	AP to OB
	mAP-OB	5.3	12.6	28.7	
	mLC-OB	6.0	13.1	29.3	LC to OB
	mOB-LC	3.3	9.9	26.7	
North-east and Orkney	mNHA-K	5.3	12.3	28.7	NHA to K
	mK-NHA	2.7	9.5	26.0	
	mNHA-NHJ	0.0	5.6	22.0	NHJ to NHA
	mNHJ-NHA	14.7	22.7	40.0	
	mDF-NHA	12.0	19.7	36.7	DF to NHA
	mNHA-DF	2.0	8.1	24.7	
	mK-NHJ	7.3	14.7	30.0	NHJ to K
	mNHJ-K	9.3	17.1	33.3	
	mDF-K	10.0	17.4	34.0	DF to K
	mK-DF	8.7	16.5	32.7	
	mDF-NHJ	5.3	12.9	28.7	NHF to DF
	mNHJ-DF	9.3	17.1	33.3	

		Number of immigrants generation^{-1} into receiving population			
Region	**Migration direction (sink-source)**	**2.5% percentile**	**Mean**	**97.5% percentile**	**Directional bias**
Shetland Islands	mUS-HS	0.0	11.8	29.3	HS to US
	mHS-US	4.0	23.5	43.3	
	mUS-CN	0.0	11.3	27.3	CN to US
	mCN-US	1.3	18.1	34.7	
	mHS-CN	0.0	11.8	30.0	CN to HS
	mCN-HS	0.0	13.6	29.3	
Isle of Man	mRB-POA	0.0	16.9	34.0	RB to POA
	mPOA-RB	0.0	14.6	30.7	
	mRB-LN	0.0	10.9	27.3	RB to LN
	mLN-RB	0.0	6.2	24.7	
	mPOA-LN	0.7	16.9	34.0	POA to LN
	mLN-POA	0.0	11.0	26.7	

Table 3.18. Mean migration rate (μ*m*) (±SD) and sum migration rate (∑*m)* from all other *M. modiolus* reefs, where *m* is number of immigrants generation^{-1} into receiving population (sink).

Receiving population (sink)	**μ*m***	**±SD**	**∑*m***
Appin	13.56	4.86	188.00
Loch Creran	11.31	3.77	167.33
Oban Bay	13.25	4.16	184.00
Karlsruhe	14.79	4.00	197.33
Noss Head (adults)	14.42	5.57	198.00
Noss Head (juveniles)	10.72	3.51	162.67
Dornoch Firth	17.53	12.51	240.67
Hascosay Sound	13.10	4.01	181.33
Uyea Sound	14.94	5.41	202.00
Calback Ness	14.72	3.80	198.00
Point of Ayre	13.49	4.25	186.67
Ramsey Bay	11.99	4.09	173.33
Little Ness	13.30	4.62	187.33

Table 3.19. Mean migration rate (μ*m*) (±SD) and sum migration rate (∑*m*) between regions, where *m* is the mean number of immigrants generation^{-1} from each population within a given region into the sink region. Directional bias determined as the migration direction which resulted in a greater migration rate. *n/a=not applicable*

Source Region	Sink Region	μ*m*	±SD	∑*m*	Directional Bias
West Coast	West Coast	11.94	1.45	71.66	*n/a*
North-east and Orkney	North-east and Orkney	22.66	4.77	188.08	*n/a*
Shetland Islands	Shetland Islands	15.01	4.87	90.05	*n/a*
Isle of Man	Isle of Man	12.76	4.19	76.57	*n/a*
North-east and Orkney	West Coast	12.33	3.85	147.94	To North-east and Orkney
West Coast	North-east and Orkney	15.76	12.55	189.08	
Shetland Islands	West Coast	16.24	5.37	146.14	To West Coast
West Coast	Shetland Islands	13.63	1.83	122.69	
Shetland Islands	North-east and Orkney	17.19	4.44	189.10	To North-east and Orkney
North-east and Orkney	Shetland Islands	14.58	5.30	174.95	
North-east and Orkney	Isle of Man	12.35	4.24	148.16	To Isle of Man
Isle of Man	North-east and Orkney	10.28	3.62	123.33	
West Coast	Isle of Man	11.42	3.90	102.82	To Isle of Man
Isle of Man	West Coast	10.19	2.83	91.74	
Shetland Islands	Isle of Man	15.32	4.33	137.84	To Isle of Man
Isle of Man	Shetland Islands	13.94	5.22	125.48	

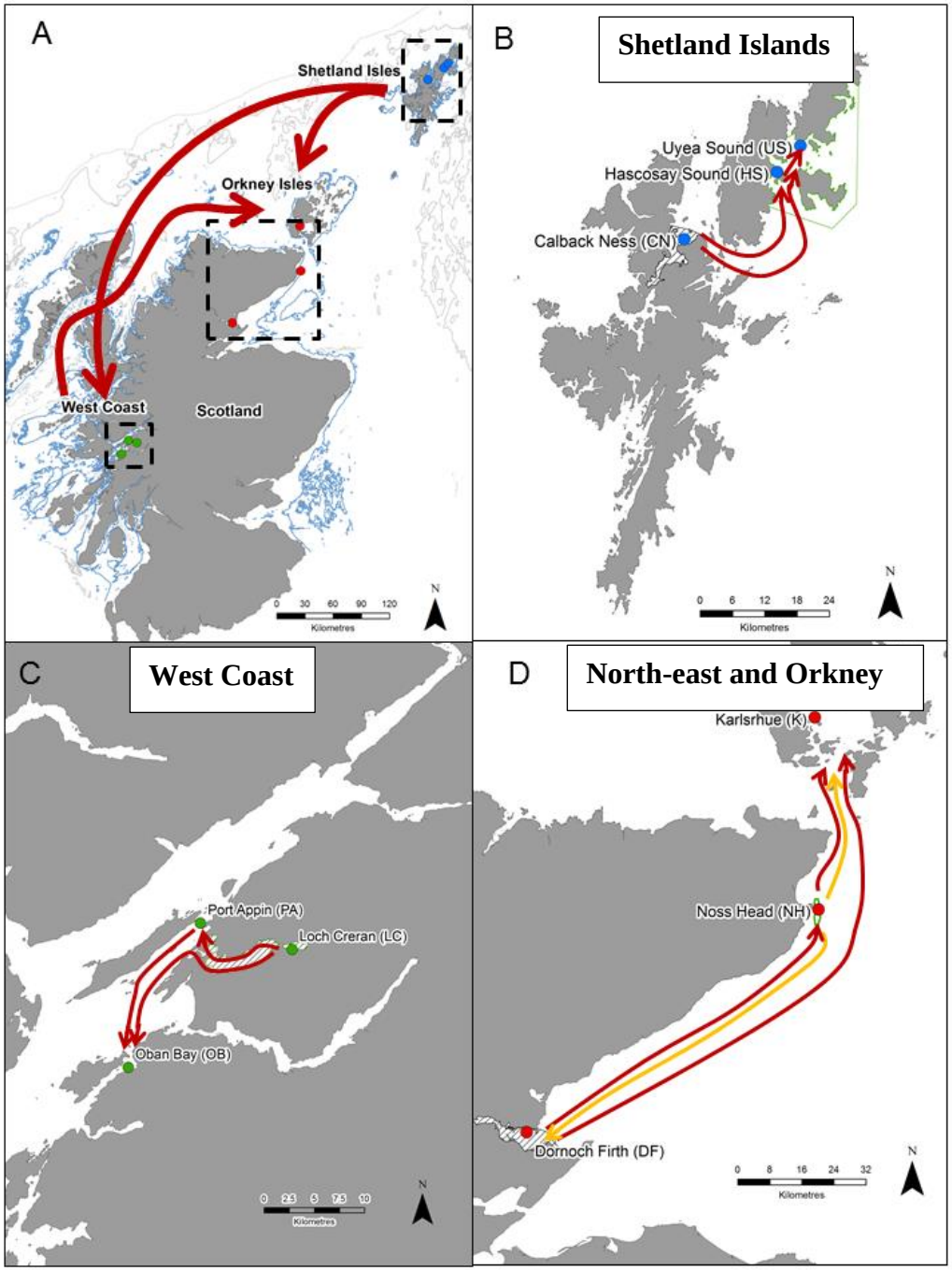


Figure 3.12. Directional bias of migration between regions (A) and between sites within Shetland Islands region (B), the West Coast region (C), the North-east and Orkney region (D). Directional bias is indicated by red and yellow arrows, where red represents adult migration and yellow indicates juvenile migration from Noss Head population. *Note: Results indicate the direction of higher migration rates only. They do not indicate uni-directional migration as migration was strong in both directions for most populations/regions.*

3.6 Discussion

Data arising from this investigation represent initial findings regarding the connectivity and genetic structure of UK and IOM *M. modiolus* reef populations and as such is a valuable contribution to current understanding of *M. modiolus* reef population genetics.

Genetic Connectivity

In general, there appears to be moderate to strong connectivity of *M. modiolus* reefs across the UK and IOM range of the habitat. This is backed-up up by both low F_{st} scores (i.e. low differentiation) and results of the STRUCTURE analyses that suggest two genetic groups with no clear spatial divide (i.e. minimal genetic structure). Additionally, mean migration values and variability in migration rates were generally comparable across sites and regions. Migration rates also indicate adequate gene flow between populations to prevent genetic differences between populations. For comparison, Palumbi (2003) reported that even one migrant per generation can have considerable influence on the genetic make-up of the sink population and as low as ten migrants per generation may be ample to prevent any gene-frequency differences between populations. Thus, it may be concluded that migration rates are high between populations and sufficient for ensuring genetic similarity (and diversity) across populations.

Distance does not appear to be a key driver of connectivity for UK and IOM *M. modiolus* reefs. The initial broad-scale examination of connectivity showed low levels of differentiation across the whole UK region, despite distances of >1000km between populations. Similarly, the connectivity analyses of Scottish populations showed both moderate differentiation across small spatial scales and minimal differentiation across larger spatial scales. For example, within the West Coast region, the Port Appin population was found to be moderately differentiated to both Loch Creran and Oban populations, despite being in close vicinity of one another. Likewise, IOM populations were found to be moderately differentiated, despite relative proximity to one another (i.e. <100 km total distance). Conversely, populations of the West Coast region were less differentiated from Shetland Islands' populations than with closer North Coast and Orkney populations.

Ocean processes are largely responsible for controlling gene flow and therefore a large driver behind shaping population networks and creating distinct source and sink

populations, or isolated populations reliant upon self-recruitment (Palumbi, 1994). Currents and tidal flows, seasonal front systems (e.g. thermoclines) and boundary layers may promote gene flow between areas or alternatively, act as invisible barriers which restrict or hamper larval movement including imposing seasonal and directional limitations (Hohenlohe, 2004; Robins *et al*., 2013; Adams *et al.*, 2014). Additionally, knowledge of local demographics, larval biology and ecology, estimates of reproductive success of immigrants and residents, and geographical influences can also improve understanding of the mechanisms driving genetic connectivity of *M. modiolus* reefs (Selkoe & Toonen, 2011). Such influences are discussed in the context of the genetic connectivity of UK and IOM *M. modiolus*.

Hydrodynamics

Knowledge of local and regional hydrodynamics including current flows, tidal regimes, and front systems is vital for understanding the forces that promote or block larval dispersal and therefore directly advance or hamper gene flow between sites. Around the UK, these forces vary immensely and consequently timing, direction and success of larval dispersal from and to *M. modiolus* populations is very much location dependent. For example, Figure 4.4 provides an overview of mean spring peak flow occurring across the Irish Sea region. Hydrodynamic characteristics of the Irish Sea also include strong tidal currents at the north and south entrances (1.0-1.5 m/s) and around headlands (2.0 m/s), weak currents in the western extent, and a generally northward baroclinic flow (Olbert *et al*., 2012). Consequently, one would potentially expect greater dispersal in populations located near entrances or headlands of the Irish Sea, less differentiation in Northern populations, and high levels of self-recruitment and differentiation in western populations. Such patterns could partially account for the increased differentiation of the Strangford Lough and Ards populations from counterpart Irish Sea populations. However, the influence of hydrodynamics is complex and the relationship between such forces and larval connectivity is not clearly understood. For example, while reefs situated in open areas of high flow that are exposed to large scale flow patterns might have improved dispersal and acquisition of larvae, long-range dispersal "success" may also lead to "dispersal dilution" and therefore decreased recruitment in sink populations. Likewise, while exposed sites might enjoy increased delivery of larvae, they may also experience problems with retention of larvae as compared to enclosed bays (Adams *et al*., 2014). Consequently, around the Isle of Man, where mean spring peak flows are

relatively high (Figure 3.13) (ABPmer, 2017), it is possible that larvae might be dispersed fairly quickly, thereby reducing potential for recruitment to nearby adjacent sites. This could explain the moderate levels of differentiation and separate genetic groups observed across IOM populations, despite close proximity to one another.

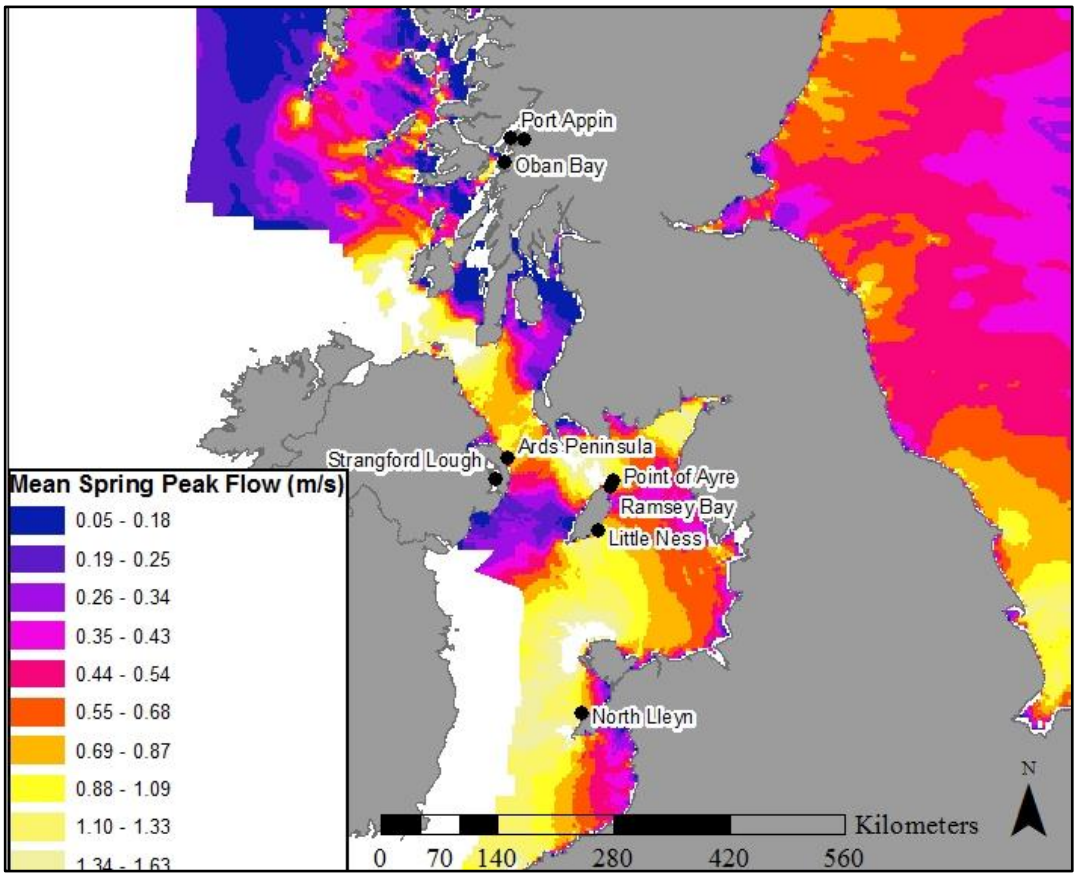


Figure 3.13. Mean spring peak flow (m/s) across the UK and IOM (Source: ABPmer website).

Past studies have combined hydrodynamics modelling (i.e. particle transport models) with genetic techniques to provide improved understanding of actual connectivity for species with a planktonic larval stage (Schunter *et al.*, 2011; Selkoe & Toonen, 2011; Coscia *et al.*, 2012). Co-consideration of both factors can also act as a means of validating either approach and therefore strengthen conclusions regarding actual levels of connectivity between sites. Results from the preliminary Irish Sea analyses conducted here contributed to Gormley *et al.* (2015) which included hydrodynamic modelling of larval dispersal from the same populations as were analysed for levels of genetic

differentiation. Simulations of larval dispersal over 30-day dispersal periods showed strong connectivity between North Ireland sites (Strangford Lough and Ards Peninsula) and confirmed potential for larval dispersal up to 150 km. Further, dispersal modelling suggested that connectivity between Northern Ireland sites and IOM and Wales sites was unlikely, therefore confirming the results of the genetic analyses. However, while genetic analyses suggested connectivity between North Lleyn and Point of Ayre sites, the dispersal model showed this to be unlikely, and thus highlights the complex nature of population connectivity and potential limitations of either/both approach(es).

Consideration of the interaction of spatial variation in hydrodynamics and larval behaviour is key to determining larval dispersal potential (Robins *et al.*, 2013). For example, marine larvae demonstrate various swimming behaviours (e.g. vertical migration) which allow them to benefit from or resist water currents, and thus have some influence over dispersal distance and settlement area (Adams *et al*., 2014; Robins *et al*., 2013). Currently, understanding of *M. modiolus* larval behaviour and development is poorly understood (refer to p. 107: *Larval Biology and Ecology*). For example, the spawning season of *M. modiolus* is not well defined, and can vary greatly with depth and latitude (likely as proxies for water temperature) (Holt *et al.*, 1998; Roberts *et al.*, 2011). Consequently, where larval biology and ecology is poorly understood or ignored entirely, hydrodynamic models have limitations.

Where previous hydrodynamic modelling of *M. modiolus* reef connectivity has considered larval behaviour, specific model parameters have varied significantly with regards planktonic period and time of year, typically to reflect a specific reef(s) of focus and therefore results are possibly irrelevant to other populations. For example, hydrodynamic modelling completed for Northern Ireland populations by Elsäßer *et al.* 2013 were run to simulate settlement times of 56 days and an autumn gamete release, as characteristic of Strangford Lough *M. modiolus*. However, results did show that the remnant Strangford Lough *M. modiolus* beds are largely self-sustaining and have limited connectivity with other Irish Sea populations, which supports results here. Conversely, Gallego *et al.* (2013) used a biophysical model to predict the connectivity between *M. modiolus* reefs in Scotland with the assumption that horse mussels spawn all year round with a settlement window of 30-40 days. The potential dispersal of offspring from this model was widespread yet connectivity potential between MPAs was determined to be

low, counter to results here. However, this model was run at a coarse scale, and therefore did not take into account nearshore reefs (e.g. in sea lochs, where a number of *M. modiolus* reefs are located), and used only a small (i.e. 3) number of release sites. Neither model was validated with genetic differentiation data.

Larval Biology and Ecology

In many marine invertebrate species, levels of self-recruitment and connectivity are highly dependent on the transport of larvae, particularly direction and magnitude of larval movement (Robins *et al.*, 2013). Consequently, understanding the influence of larval biology including such aspects as growth and development, metabolism, mortality rates, health and disease, and life history traits to larval transport is a key consideration when examining the role of larval dispersal in shaping genetic connectivity. The length of time that larvae remain in the plankton, for instance, is chiefly dependent on factors such as development mode (planktotrophic versus lecithotropic), accumulated energy reserves, and time required to become metamorphically competent (Jaeckle, 1994). For example, the blue mussel (*Mytilus edulis*) typically has a larval developmental period of approximately 30 days but can persist in the plankton beyond 2 months, with development time largely correlated with sea temperature (Bayne, 1976). Where larvae have a reduced developmental period, dispersal time (and therefore dispersal distance) may be reduced, reducing gene flow potential between populations that are situated over large distances or have temporally occurring hydrodynamic processes acting as restrictions to movement. Conversely, where larvae are slow-growing and spend substantial time in the plankton, greater dispersal distances may be achieved potentially leading to greater recruitment success as larvae are able to reach increased number of populations. Results here suggest that genetic connectivity of *M. modiolu*s populations can occur over sizable distances (i.e >500 km) and therefore it is possible that *M. modiolus* larvae can spend considerable time in the plankton due to an extended developmental period. However, it may also indicate the presence of stepping-stone populations that support the transfer of genetic material over great distances.

The role of larval ecology to dispersal and recruitment success is also an important consideration when assessing influences on genetic connectivity. Ecological aspects, such as the effects of temperature and algal concentrations (i.e. feed availability) to larval development and behaviour, could progress understanding of dispersal potential and

recruitment success (Levin, 2006). Likewise, knowledge of larval traits that allow for survival to settlement and the roles of post-settlement competition and predator-prey interactions in recruitment success may be key to fully comprehending the mechanisms driving genetic connectivity. This is particularly important over a wider spatial scale as such ecological interactions are influenced by physical conditions occurring at source and sink population sites, as well as larval pelagic conditions (Cowen & Sponaugle, 2009). Consideration of ecological data could aid in explaining why distance appears to be a lesser influence on the genetic connectivity of *M. modiolus* beds. For example, the high migration rate observed between relatively distant populations (e.g. Loch Creran to Dornoch Firth) could be due to favourable larval conditions occurring at the latter leading to increased levels of recruitment.

However, current knowledge and understanding regarding the *M. modiolus* reproduction, larval biology and ecology remains limited. Research that does exist suggests that the reproductive cycle of UK M. modiolus lacks seasonal synchronicity with recruitment, spawning period, developmental period and gonadal maturation varying across seasons, year, depth and location (Holt *et al.*, 1998; Roberts *et al.*, 2011; Dinesen & Morton, 2014). Roberts *et al.* (2011) provide a detailed overview of larval development (including the effect of diet) for the species but investigation was confined to the Strangford Lough population. As larval growth and development is largely driven by local conditions such as feed availability and temperature (Bertram & Strathmann, 1998; Cowen & Sponaugle, 2009), results (e.g. developmental time) may not apply to other populations. This poses a considerable challenge to hydrodynamic modelling in that information based on a particular population or particular set of experimental conditions may not be relevant when applied across a wider spatial scale.

Local Demographics

In addition to hydrodynamic influences, local demographics have bearing on reproduction rates, dispersal, and recruitment success and therefore are likely to influence genetic connectivity of sites. Brash *et al*. (2017), for example, examined juvenile abundance and reef density in relation to current flow in *M. modiolus* reefs across the UK distribution (including North Lleyn, Port Appin and Karlsruhe reefs). The Karlsruhe reef was observed to have decreased abundance of juveniles and lower densities than other reefs, with both variables associated with reduced tidal flow. Reefs with increased

densities tend to have higher structural complexity which can aid in recruitment via providing shelter to juveniles from predation (Comely, 1978; Holt *et al*., 1998) and by increasing post-settlement survival (Gutierrez *et al*., 2003; Nestlerode *et al*., 2007). Similarly, Elsäßer *et al.* (2013) noted that higher reef elevation (which could be associated with density) in Strangford Lough population resulted in larvae being able to disperse to greater distances. Consequently, the decreased density, recruitment and current flow observed at the Karlsruhe reef suggest it could be relatively isolated from other reefs and could partially explain the observed levels of moderate differentiation as compared to other Scottish *M. modiolus* reefs. STRUCTURE results also indicated that Karlsruhe was of a different genetic group than nearby northern sites on both the Scottish mainland and in Shetland Islands, further supporting this argument.

The Port Appin population was also moderately differentiated from other populations. As high levels of juvenile abundance have been recorded at this bed (Brash *et al*., 2017), results potentially indicate increased levels of self-recruitment. However, given the high current flow associated with the site (Brash *et al.,* 2017), it is more likely that the population is recruiting from an unsampled or unknown site, particularly given that investigation was limited to a low number of sites for the region. For example, there are a number of other west coast horse mussel populations (e.g. Loch Alsh and around the Small Isles) that may act as larval sources. Likewise, there may be undiscovered beds that supply the Port Appin and Karlsruhe beds, thus accounting for their separation as a unique genetic group.

Coastline Geography

The dispersal stage of a species with sessile adults (such as horse mussels) must select sites that are environmentally suitable for establishment and survival, but must also be able to reach such locations. Consequently, where local coastline features such as headlands, narrows and sea lochs directly influence accessibility (including dispersal and retention), such geographies may have a considerable influence on larval connectivity and therefore genetic connectivity of sites (Robins *et al*., 2013; Adams *et al.*, 2014). For example, investigation into the role of local geography to dispersal carried out by Adams *et al.* (2014) showed that dispersal success was largely dependent on current velocity, as previously discussed, but also coastline geography (e.g. wave fetch, openness) and habitat availability. Particles released from regions of high current velocity, open coastline and

low local habitat availability (e.g. headlands, islands) travelled furthest but were less likely to disperse successfully to other coastal sites as compared to those living along sheltered and enclosed coasts (Adams *et al.*, 2014). Additionally, nearshore processes such as flood-tide asymmetries (produced by coastal morphology) influence self-recruitment and connectivity (Robins *et al*., 2013).

Elsäßer *et al.* (2013) demonstrated the effect of local coastline to recruitment showing that reduced connectivity between Irish Sea horse mussel beds and Strangford Lough populations was a direct consequence of larvae's inability to move beyond Strangford Narrows. While the processes impacting the Strangford Lough bed might be expected to occur for Scottish sea lochs, the presence of narrows (characteristic of loch environments) with high current flow may also aid in larval dispersal to other sites (Robins *et al*., 2013). Likewise, while high currents around headlands and other exposed sites might promote larval dispersal, the same conditions can lead to larval dilution, particularly in species with extended larval duration, and thus reduce settlement and recruitment success (Adams *et al.*, 2014). Such processes could explain the moderate differentiation of the exposed Noss Head bed from all other Scottish beds. Further, the low differentiation between Noss Head adults and juveniles could also indicate that the bed is largely self-recruiting. However, STRUCTURE analysis suggests that the Noss Head juveniles include individuals of a separate genetic group, thereby suggesting an additional external source of larvae.

Genetic Diversity

All *M. modiolus* populations had lower H_o than H_e and positive F_{is} values indicating a possible heterozygote deficiency and suggesting that all populations were not in HWE. HWE states that any genetic variation in a population will stay constant over generations in the absence of disturbing factors such as gene flow, natural selection or mutations (Lowe *et al*., 2004). Consequently, deviation from HWE, where observed heterozygosity is different than expected heterozygosity, indicates that a factor such as inbreeding (if Ho<He, as seen here) or mixing of two previously isolate populations (if He<Ho) must be considered (Lowe *et al.*, 2004). Null alleles were detected across all markers and populations and may account for the observed heterozygote deficiency. Null alleles occur when a primer is prevented from annealing to template DNA during amplification of the microsatellite locus by PCR and can lead to under-estimation of genetic diversity

(Chapuis & Estoup, 2007). They are a relatively common occurrence in mollusc species (Reece *et al*. 2004; Mariani *et al.* 2012; Coscia *et al.*, 2012).

Addison & Hart (2005) also found that species with planktonic reproduction (e.g. broadcast spawners with external fertilisation) tend to have greater heterozygote deficits and therefore increased departures from HWE. This may be due to factors such as increased mutation rates arising from the high number of cell cycles needs to produce excess gametes or the Wahlund effect (i.e. coexistence of genetically distinct cohorts within a sampling location) (Addison & Hart, 2005; Coscia *et al*., 2012). Additionally, large variation in reproductive success may lead to effective population (i.e. the cohort of the population that reproduces) sizes that are much smaller than census numbers, and consequently lead to reduced heterozygosity across a population (Bierne *et al.,* 1998).

In their review of F_{is} values in marine species, Addison & Hart (2005) reported a mean F_{is} of 0.149 across 89 studies of species with planktonic larval dispersal. Comparatively, F_{is} scores for *M. modiolus* reefs as measured here seem high. However, it has been reported that high F_{is} scores are typical of bivalve species (Addison & Hart, 2005). Further, while positive F_{is} values may indicate inbreeding (which could lead to adaptive decline in these populations) it is argued that this is unlikely to be occurring in *M. modiolus*. Instead the high F_{is} scores are more likely to be a consequence of the heterozygote deficiency commonly observed in marine bivalves, as previously discussed.

While absolute values of diversity do not seem reliable here, for reasons discussed above, a relative comparison of metrics may at least illustrate variation in the genetic diversity between *M. modiolus* populations. In general, IOM populations tended to have higher H_o and H_e values and lower F_{is} scores than Scottish populations, potentially indicating greater genetic diversity in IOM populations. Given that directional bias of migration from all Scottish regions was towards IOM populations, this could account for the increased diversity observed in the southern populations. Further, Ramsey Bay (IOM) had the highest H_o, highest number of alleles, greatest allelic richness score and one of the lowest F_{is} scores across all IOM and Scottish poulations, suggesting it could be an important sink population. Migration rates within the IOM also suggest that Ramsey Bay may support both Point of Ayre and Little Ness sites. All populations of the Shetland Islands had similarly high allelic richness scores and low F_{is} scores while northern (Noss Head and

Dornoch Firth) populations and western (Loch Creran and Oban Bay) populations showed similarly low H_o scores and increased F_{is} scores. Karlsruhe and Port Appin populations showed similar diversity indices, which may be a consequence of being part of the same genetic grouping.

Adaptive Capacity

Given that population connectivity analyses indicated moderate to high levels of gene flow across all horse mussel populations, one would expect that adaptive capacity is generally limited within populations at a site level (Sanford & Kelly, 2009) (i.e. reduced potential to adapt to site-specific stress conditions). For example, more southern or shallower populations may struggle to adapt to warming conditions if they are sinks for larval supply from northern/deeper (i.e. cooler) environments. Reduced adaptive capacity has historically been presumed for bivalve species owing to having planktonic larvae and therefore widespread dispersal. However, differentiation and adaptation have been observed in other bivalve species, and over a range of spatial scales (i.e. meters to kilometers), so should not be ruled out (Kuo & Sanford, 2009). Likewise, increased genetic connectivity may support higher levels of genetic diversity which may contribute to improved resilience by providing a diverse gene pool from which natural selection may occur (Sanford & Kelly, 2011).

It is also important to note that genetic differentiation is not a critical precondition for adaptation as populations that appear homogeneous for neutral loci (such as microsatellites) may still exhibit adaptation (Sanford & Kelly, 2011). Furthermore, in addition to gene flow and natural selection, a number of other intra-populational factors may also influence potential for adaptation. For example, gene mutation and random events such genetic drift where allele frequencies will vary due to chance sampling events may both play a role (Hellberg *et al*., 2002; Palumbi, 2003).

From the fossil records, there is an indication that rates of speciation of marine animals coincided with changes in environmental CO_2 (Cornette *et al.,* 2002) suggesting that if species are able to adapt sufficiently quickly to keep pace with changing environmental conditions, impacts should be lessened. Conversely, the relatively long generation length of many bivalve species makes it is unlikely that these organisms will be able to undergo rapid evolution to keep up current increasing rates of climate change (Kurihara, 2008).

This is particular relevant to *M. modiolus* which are notably slow growing with an extended lifespan (Halanych *et al*., 2013; Fariñas-Franco *et al.*, 2014).

Future climate change could alter genetic connectivity of *M. modiolus* populations, and therefore potentially lead to increased variation in genetic structure between populations. For example, increased temperature may cause changes in reproduction, spawning, embryonic and gonad development of benthic species, and therefore lead to changes in the timing of *M. modiolus* lifecycle events (Birchenough *et al*., 2015). Likewise, changes in hydrodynamic regimes (e.g. circulation patterns) could alter larval movement in the water column or cause a mismatch between larvae and their food source (phytoplankton). Consequently, such impacts could dramatically alter larval dispersal and recruitment success (Birchenough *et al*., 2015). Any change in such factors would have knock-on effects to population genetic structure and therefore connectivity, diversity and adaptive capacity.

Regardless of whether adaptation is able to occur in *M. modiolus*, the influence of genetic factors on resilience to climate change should not be disregarded. *M. modiolus* may still be capable of adjusting gene expression (for example, by alteration of transcription or translation) to attain physiological plasticity in response to physical stressors in their environments (Hofmann & Todgman, 2010; Whitehead, 2012), particularly where populations have historically experienced variable or extreme conditions (refer to Chapter 2). Likewise, as physiological acclimatisation is associated with increasing genetic differentiation between populations of the same species, where moderate differentiation was observed, increased plasticity may also be observed (Pörtner, 2002). Any variation in stress responses of populations (refer to Chapter 4) may indicate genetic differences in populations' abilities to mount responses to climate change stressors.

4. **Sensitivity and Acclimation Potential:** Thermal limits and oxidative stress response in *M. modiolus* under climate change conditions

4.1 Abstract

An understanding of *M. modiolus'* sensitivity to stress conditions is critical for accurate determination of how the species will fare under climate change conditions. As a bivalve, the species has a poor thermal regulatory ability and therefore may have decreased acclimatory ability under warming conditions. Likewise, as the species predominantly inhabits subtidal environments that are characterised by relatively stable conditions, it may be further sensitive to a changing environment. Examination of physiological limits will provide key information on the thermal tolerance of the species, and consideration of multiple populations may highlight differences in tolerance abilities that exist across the species' range. Further, examination of physiological responses under climate change may provide evidence on the species' acclimatory ability under stress conditions. In this chapter, thermal limits were investigated to provide an indication of the degree of temperature change that *M. modiolus* may be able to tolerate and to examine whether populations from across a latitudinal gradient may have varying thermal ranges as suggested by historical exposures. Results of thermal limits experiments show some variation between populations' thermal ranges and additional consideration of the influence of hypoxia to thermal tolerance suggests that hypoxic conditions may contract the thermal tolerance range of *M. modiolus*. Additionally, this chapter investigates oxidative stress response in *M. modiolus* under a series of varying exposures to warming and/or hypoxia with consideration of both species- and population-level effects. Results suggest that populations may have varying oxidative stress responses and that *M. modiolus* is likely more sensitive to thermal stress than hypoxia. Results are discussed in

the context of climate change vulnerability with consideration of a range of factors including physiology and population demographics.

4.2 Aims and Objectives

Aims:

- Examine the sensitivity and acclimatisation potential of *M. modiolus* to climate change stressors including warming and hypoxia;

- Examine thermal limits and oxidative stress response in *M. modiolus* populations under climate change conditions (warming and hypoxia) to determine species- and population-based responses.

Key Objectives:

- **Compare thermal limits** of *M. modiolus* in populations from across the UK species' range;

- **Investigate impact of a co-stressor (hypoxia) to thermal limits** in *M. modiolus*;

- **Compare thermal and hypoxic acclimation abilities** of *M. modiolus* populations from across the UK via series of short term (week duration) and longer term (month duration) exposure experiments;

- **Examine oxidative stress response** including damages to lipid, proteins and DNA and production of antioxidants in *M. modiolus* exposed to climate change conditions.

4.3 Introduction

Key to accurately forecasting how any species will fare under global climate change conditions is an understanding of a species' physiological sensitivity and ability to buffer environmental change (Hofmann & Todgman, 2010). Sensitivity refers to the degree to which an organism is adversely or beneficially effected by a stimulus and indicates the "dose-response" relationship between exposure to a stressor and resulting impacts (Füsssel & Klein, 2006). Temperature sensitivities of ectotherms vary across species and populations, and are dependent on latitude and seasonal temperature variation (Pörtner *et al*., 2005). Likewise, variation in physiological capacities to compensate for change (i.e. acclimatisation ability) occurs among and within species, and can vary over time in response to predictable environmental fluctuations (i.e. daily/seasonal changes) and over spatial scales in response to environmental gradients (Sanford & Kelly, 2011).

Bivalves are poikilothermic (i.e. unable to thermo-regulate), considered poor regulators of haemolymph acid-base balance, and may be sensitive to changes in salinity (Michaelidis *et al.,* 2005; Whiteley, 2011; Halanych *et al*., 2013). Consequently, species such as *M. modiolu*s may be highly sensitive to changing oceanic conditions and therefore directly threatened by climate change. This may be exacerbated because *M. modiolus* is largely a sub-tidal species and therefore accustomed to relatively stable seawater temperature and pH conditions, as compared with intertidal species. Consequently, the species may be less physiologically capable (i.e. possess reduced plasticity) to deal with seawater changes and therefore may become increasingly vulnerable as climate induced changes in marine environments increase. Any such effects could yield serious consequences for the biogeography of species and thus, will be important considerations for management of the species (Heath *et al.,* 2012)

The effects of thermal stress to *M. modiolus* are of particular concern. Temperature influences the physiology of ectotherms at all levels of biological organisation from molecules to cells, tissues and the whole animal, influences the rate of biological processes and alters the structural and functional properties of macromolecules (Whiteley & Mackenzie, 2016). For example, it has been proposed that a 2°C increase in temperature could accelerate physiological processes by as much as 30% (Gruber, 2011).

Thermal Limits

A species may exist across a range of temperatures, commonly referred to as its thermal tolerance range. Thermal limits refer to the temperature tolerance limits of a species' physiological processes under warming or cooling stress and are defined by upper and lower critical temperatures, beyond which survival is unlikely (Pörtner, 2002). As climate change will alter temperature extremes and averages in marine environments, there is particular interest in determining species' thermal limits so as to predict whether such changes will force species close to or beyond their limits (Tomanek, 2012). Further, knowledge of thermal limits may also provide insight regarding potential shifts in biogeographic distribution ranges under climate change conditions (Tomanek, 2008; Tomanek, 2012). Additionally, physiological acclimatisation to varying temperature ranges (e.g. in populations situated over a latitudinal gradient) correlates with increasing genetic differentiation (Portner, 2002) so may have consequences for population genetic structure (refer to Chapter 3).

Thermal tolerance windows are set by the limited capacity of oxygen supply (Frederich & Pörtner, 2000; Pörtner *et al.*, 2005) (Figure 4.1). At temperatures approaching a species' thermal limits, excessive oxygen demand in peripheral tissues causes insufficient oxygen levels in the body fluids and limitation of oxygen supply to central tissues, producing thermally induced hypoxia (Heise *et al*., 2006). A loss in aerobic scope and progressive internal hypoxia define the onset of thermal limitation, and such conditions lead to cellular and molecular disturbances and the onset of anaerobic metabolism (Zielinski & Pörtner,1996; Pörtner, 2002).

Furthermore, while warming contributes to internal hypoxia within tissues and body fluids, this may be exacerbated by low-oxygen concentrations in external environments (i.e. deoxygenation zones, hypoxic events) as predicted under climate change (Keeling *et al*., 2010). Accordingly, climate change conditions of warming and hypoxia may have parallel or synergistic impacts to thermal limits. Intra- and interspecific variation in thermal tolerance limit and thermal tolerance ranges has been well illustrated along spatial gradients (e.g. latitude) (Whiteley & Mackenzie, 2016), and thus comparison across populations may shed light on respective sensitivities.

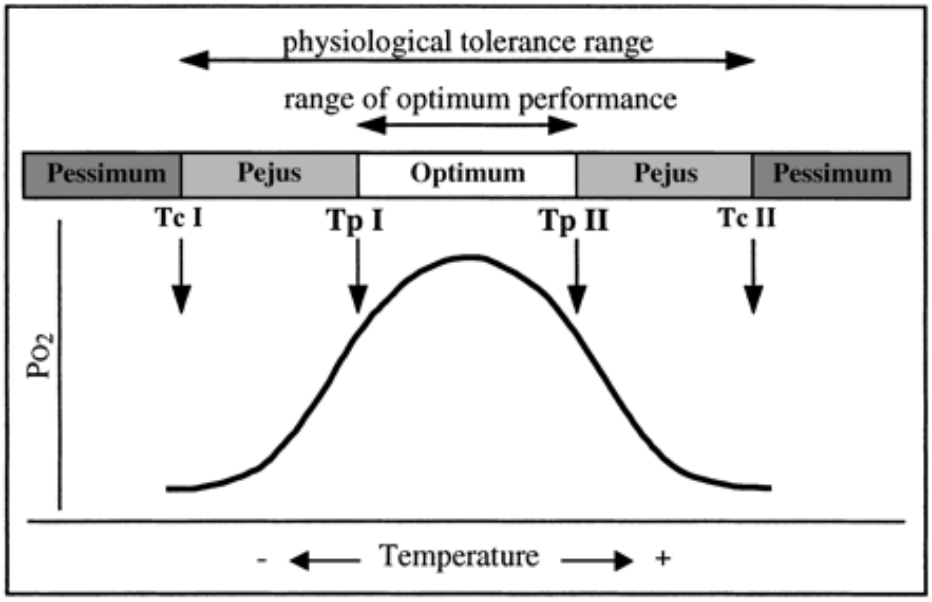


Figure 4.1. Temperature tolerance model illustrating the relationship between thermal tolerance and internal O2 levels (pO2). Critical temperatures (Tc) indicate a transition to anaerobic metabolism and therefore sets the physiological tolerance range. Pejus temperatures (Tp) indicate a transition from optimum to pejus (worsening) temperature range and therefore set the optimum performance range (Source: Frederich & Pörtner, 2000).

Oxidative Stress

As thermal limits are approached, increased demand for oxygen to support physiological processes (e.g. metabolism) leads to oxidative stress. Oxidative stress arises due to the 'Oxygen Paradox' referring to the fact that while eukaryotic aerobic organisms cannot exist without oxygen, oxygen is inherently dangerous to their existence (Ahmad, 1995; Davies, 1995). Ultimately, oxidative stress results in a state of unbalanced tissue oxidation and disturbed cellular redox activity (Abele & Puntarulo, 2004) leading to potentially lethal impacts for any given organism. The oxidative stress response refers specifically to the production and accumulation of reduced oxygen intermediates in response to exposure to environmental stressors including thermal stress, ultraviolet radiation or pollution (Ahmad, 1995; Lesser, 2006).

The innate toxicity of oxygen is directly attributed to its chemical structure. Oxygen in singlet and molecular forms possesses unpaired electrons and therefore acts as a free radical with the potential to cause substantial cellular damage. During the reduction of

oxygen free radicals into water, a number of reactive and highly toxic intermediate products are produced (Ahmad, 1995; Davies, 1995). These include the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•), collectively termed oxygen free radicals or more commonly, reactive oxygen species (ROS) (Abele & Puntarulo, 2004; Lesser, 2006). Superoxide radicals are produced when free electrons pair up with single unpaired electrons of the oxygen molecules to produce a charged molecule. Once generated, O_2^- is then reduced to H_2O_2 either spontaneously or by the antioxidant superoxide dismutase (SOD) (refer to pp.121-122, *Antioxidant Defences*). If the molecule is further reduced, it produces HO•, the most reactive of the oxygen radicals (Ahmad, 1995; Lesser, 2006) (Figure 4.2).

ROS Damages

Organisms are highly susceptible to ROS, particularly H_2O_2 and HO•. H_2O_2 is a neutral molecule and as such, can readily diffuse across cellular membranes. Consequently, it can cause substantial damage because it is not restricted to its point of synthesis in the cell and is able to enter into numerous other cellular reactions. Direct damage to DNA and enzymes can occur during exposure to H_2O_2. Additionally, H_2O_2 has an extended lifetime in seawater so may pose a greater threat to marine organisms (Lesser, 2006). HO•, the most destructive of the ROS, attacks biological molecules in a diffusion-controlled manner and thus, has huge potential for causing biological damage. The high reactivity of HO• allows it to be relatively non-specific in its targets for oxidation (Sheehan & McDonagh, 2008). It can set-off free radical chain reactions, oxidise membrane lipids, and cause proteins and nucleic acids to denature (Ahmad, 1995; Lesser, 2006). The production of ROS is directly and positively related to the concentration of oxygen within the biological system. All respiring cells produce ROS and the production and accumulation of ROS beyond the capacity of an organism to reduce these reactive species can lead to lipid, protein, carbohydrate and DNA damages (Cadenas, 1995; Lesser, 2006).

Damage to lipids as a consequence of ROS reactions is one of the most common mechanisms of cellular damage. The oxidative degradation of lipids, known as lipid peroxidation, is particularly cytotoxic in mitochondria where effects include changes in enzyme activity and ATP production as well as initiation of apoptosis (Lesser, 2006). Likewise, a wide range of proteins may be damaged or degraded by ROS. This is

principally due to aggregation, fragmentation and amino acid modification by ROS (Davies, 1987). Such damages can lead to permanent loss of function and may lead to the elimination or accumulation of damaged proteins (Ghezzi & Bonetto, 2003) with knock-on effects to various biological processes and structures. A key cause of damage to proteins is carbonylation of the protein which refers to the irreversible modification of amino acid sidechains to aldehyde and ketone (i.e. carbonyl) groups which ultimately resulting in protein inactivation, aggregation and degradation (Levine *et al*., 2000).

The generation of ROS can also induce deletions, mutations and other lethal genetic effects to DNA (Lesser, 2006). Both the sugar and base components of the DNA structure are susceptible to oxidation, which causes base degradation, single strand breakage and cross-linking to proteins (Lesser, 2006). Damage to DNA is undoubtedly a major threat to organismal health and survival. Any un-programmed changes in the structure of the DNA molecule may result in serious biological conditions. As interest in the impacts of environmental stressors in marine environments increases, the level of strand breakage in DNA has been used as an effective biomarker for environmental bio-monitoring (Frenzilli *et al*., 2001).

Antioxidant Defences

To combat ROS accumulation, organisms express both low molecular mass and high molecular mass antioxidants. The former includes both water soluble (e.g. ascorbic acid) and lipid-soluble (e.g. retinol) forms; these act as free radical scavengers (Lushchak, 2011). High molecular mass antioxidants include a number of enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, which serve to inactivate ROS by reduction to water and thereby aid in preventing subsequent cellular damage (Ahmad, 1995; Lesser, 2006). SOD is a catalyst enzyme that maintains levels of superoxide anions at a steady concentration and significantly shortens the lifetime of ROS while catalase and glutathione peroxidise are enzymes that catalyse the conversion of H_2O_2 to H_2O and O_2 (Abele & Puntarulo, 2004; Lesser, 2006) (Figure 4.2). These antioxidants are generally referred to as primary antioxidants as they constitute a primary line of defence against ROS (Cadenas, 1995).

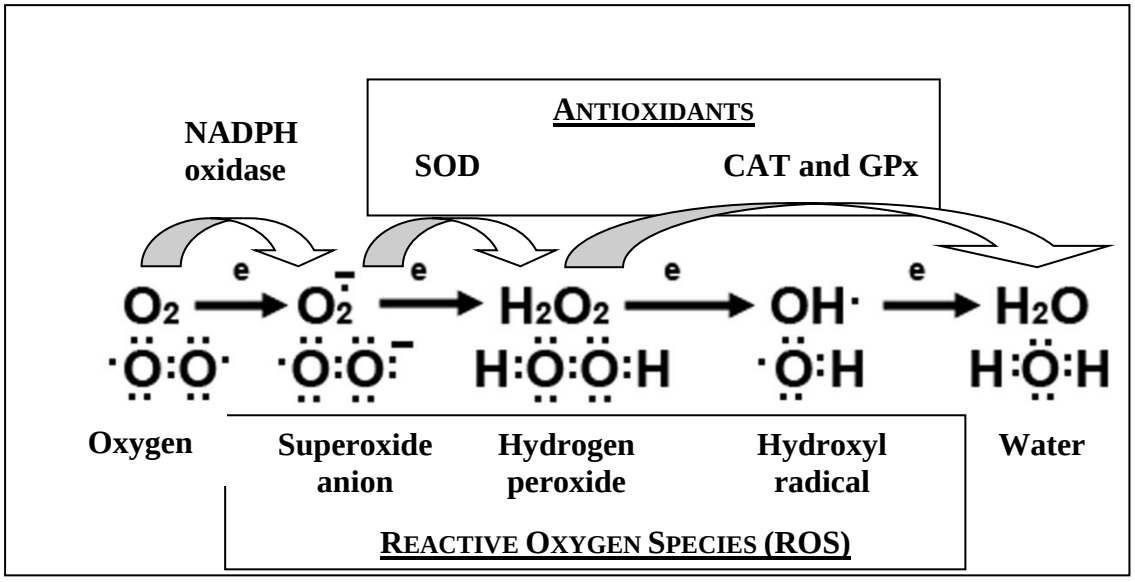


Figure 4.2. The production and reduction of reactive oxygen species. A superoxide anion is produced by the addition of a free electron to the oxygen molecule (promoted by NADPH oxidase). Following, the antioxidant superoxide dismutase (SOD) reduces superoxide to hydrogen peroxide which is then reduced to hydroxyl radical and ultimately water by the antioxidants catalase (CAT) and glutathione peroxidase (GPx) (adapted from Racila & Bickenbach, 2009).

Detection of Climate Change Impacts

Oxidative stress has emerged as a subject of increased interest with regards the impacts of global climate change on natural ecosystems. It is an important part of the stress response in marine organisms, and has to date been used to monitor the effects of changes in environmental conditions ranging from thermal stress to pollution (Lesser, 2006). Additionally, investigation of oxidative stress may be integrated with various other research approaches including molecular genetics and proteomics, thereby contributing to an integrated view of organismal responses to stressor conditions.

While higher functional levels of biological organisation (e.g. organelle, whole animal) tend to have increased thermal sensitivity as compared to cellular or molecular components (Pörtner, 2002), changes at the molecular level under stress conditions may reveal underlying responses not immediately apparent in higher level biomarkers. Similarly, they may parallel changes observed at higher levels of organisation and thus provide indication of mechanisms that enable physiological acclimation and evolutionary

adaptation to such conditions (Whitehead, 2012). For example, increases in seawater temperature towards thermal maxima may initiate oxidative stress in ectothermic species via temperature-related increases in metabolic rates leading to a rise in reactive oxygen species (ROS) production as a side product (Ahmad, 1995; Lesser, 2006; Lushchak, 2011). It is also argued that molecular or cellular approaches may be more practical for monitoring purposes, as analyses can be carried out on small amounts of tissue or blood which may be collected and preserved *in-situ*, as opposed to investigations across more complex physiological systems (i.e. oxygen consumption) or whole animals.

Oxidative Stress in Marine Ectotherms

Prior investigation into the effects of climate change stressors including temperature, salinity, and acidification has demonstrated that climate change impacts may lead to higher levels of oxidative stress in bivalve species (An & Choi, 2010; Mattoo *et al*., 2013; Matozzo *et al*., 2013; Tomanek, 2011; Tomanek, 2012). Given that a high number of ectotherms taxa (e.g. molluscs) are largely sedentary, many lack the option of migrating to more favourable latitudes or depths when exposed to thermal stress conditions, and instead must acclimate to changing conditions or perish. Additionally problematic, ectotherms are largely unable to thermo-regulate (Whiteley & Mackenzie, 2016) and thus any change in environmental temperature directly influences physiological processes. An increase in temperature, for example, stimulates all metabolic processes thereby increasing oxygen consumption and ROS production as side products of intensified metabolism (Lushchak, 2011).

Thermal stress in ectotherms have been observed to cause significant rises in the production of ROS with associated damages to proteins, lipid membranes and DNA. For example, An & Choi (2010) found that thermal and osmotic stress caused a significant rise in ROS (H_2O_2) and increased lipid peroxidation in a bivalve species, *Scapharca broughtonii.* Similarly, protein carbonylation in response to oxidative stress has been observed in marine bivalves under varying sources of stress, though typically in response to toxicological stressors. McDonagh *et al.* (2005), for example, noted carbonylation in gill and digestive gland tissues in *M. edulis* exposed to marine pollutants. The effects of climate change stressors on protein carbonylation have received considerably less research attention.

DNA damage in marine bivalves under conditions of environmental stress has been well researched though predominantly from an ectoxicological perspective (Coughlan *et al.*, 2002; Taban *et al.*, 2004; Dhawan *et al.*, 2009; Nahrgang *et al.*, 2013). For example, Coughlan *et al.* (2002) used the approach in examining the effects of chronic exposure to polluted sediments in the Manila clam, *Tapes semidecussatus*. Significant differences between control and polluted sites were found in haemocyte, digestive gland and gill tissues, the latter showing the strongest effect. Though limited, there has been some investigation into the impacts of thermal stress to DNA integrity. For example, Buschini *et al*. (2003) demonstrated increased DNA damage in the haemocytes of a freshwater bivalve, *Dreissena polymorpha* under increased temperature. Similarly, Yao & Somero (2012) observed that thermal stress (heat and cold) lead to significant damage in the DNA of *Mytilus galloprovinicialis* and *Mytilus californianus* with degree of damage dependent on time of exposure, temperature and species. Vosloo *et al.* (2013) also demonstrated that co-stressors (temperature and oxygen stress) may impact DNA integrity in bivalves, and Frenzilli *et al.* (2001) showed that mussels (*Mytilus galloprovinicalis*) had decreased DNA integrity during summer months, highlighting that the effect of seasonality when assessing *in-situ* DNA damage.

Marine ecotherm species have the ability to adjust antioxidant levels under thermal stress conditions, so may be able to mount an appropriate defence to oxidative damage. An & Choi (2010), for example, observed that changes in water temperature (±10°C from control of 20°C) significantly increased expression and activity of antioxidants (catalase and superoxide dismutase) in digestive glands and gill tissues of a bivalve species, *Scapharca broughtonii* particularly in high temperature treatments. Vasloo *et al.* (2013) also demonstrated that increased temperature lead to higher antioxidant output in a marine gastropod, *Haliotis midae*, but this was paralleled by decreased antioxidant capabilities. Matozzo *et al.* (2013) further showed that both decreased pH and increased temperatures influenced levels of antioxidants in two bivalve species, *M. galloprovinicialis* and *C. gallina*.

This chapter provides an overview of research that was carried out to investigate thermal limits and oxidative stress response in *M. modiolus* under conditions of warming and hypoxia. Firstly, thermal limits were compared in populations from across the habitat range including a northern, southern and mid-range population. Additionally, the influence of hypoxia to thermal limits was examined in a single species in order to

highlight potential effects of co-stressors to thermal tolerance limits. Next, a preliminary investigation of the impacts of acute warming (i.e. over 24 hours) to oxidative stress response was carried out for a single *M. modiolus* population. Following, the impacts of a one-week exposure to warming to oxidative stress response were compared in populations from across the habitat range including a northern, southern and mid-range population, and at temperatures representative of current and future conditions at each site. A follow-up experiment was also carried out to examine the impacts of a one-week exposure to hypoxia in the southernmost population (North Lleyn, Wales). Finally, month-long exposures to warming and hypoxia were carried out with a northern and southern population to determine potential species- and population-level effects to the oxidative stress response. Cumulatively, investigations will contribute to a stronger understanding of the potential sensitivity of *M. modiolus* to climate change conditions and provide preliminary indication that *M. modiolus* reef populations may have varying resiliencies under stress conditions.

4.4 Methodology

Experiment 1. Comparison of thermal limits in *M. modiolus* reef populations from across a latitudinal gradient

Animal Collection

Adult *M. modiolus* were hand collected directly from the seabed by members of the Heriot-Watt Scientific Dive Team from North Lleyn (Wales), Port Appin (Scotland) and Karlsruhe (Scapa Flow, Orkney) reefs in Spring/Summer 2014 (Table 4.1, Figure 4.3). Animals were placed in cold boxes with seawater and ice packs (temperature ~ 8°C) and transported to Heriot-Watt University within 24 hours of collection. Seawater oxygen levels were maintained via use of portable air lines with air stones and mortality rates were <1%. Animals were transferred to a flow-through aquarium tanks filled with re-circulated seawater and fed twice weekly with algae (water dosed with 5L of 1:1 *T-iso lutea* and *Tetraselmis suecica*). Populations were kept in separate aquarium tanks from one another and maintained at 15°C for a 4-week acclimation period prior to experimental start.

Table 4.1. Collection data (date, latitude/longitude, depth) for *M. modiolus* reef populations used in thermal limits and oxidative stress experiments. Temperature at time of collection is also provided.

Population	Collection Date	Latitude, Longitude	Depth (m)	Temperature (°C)
Karlsruhe (Orkney)	May 2014	58.891, -3.188	24	8
Port Appin (West Scotland)	June 2014	56.551, -5.424	19.5	11
North Lleyn (Wales)	June 2014	52.936, -4.650	29	14

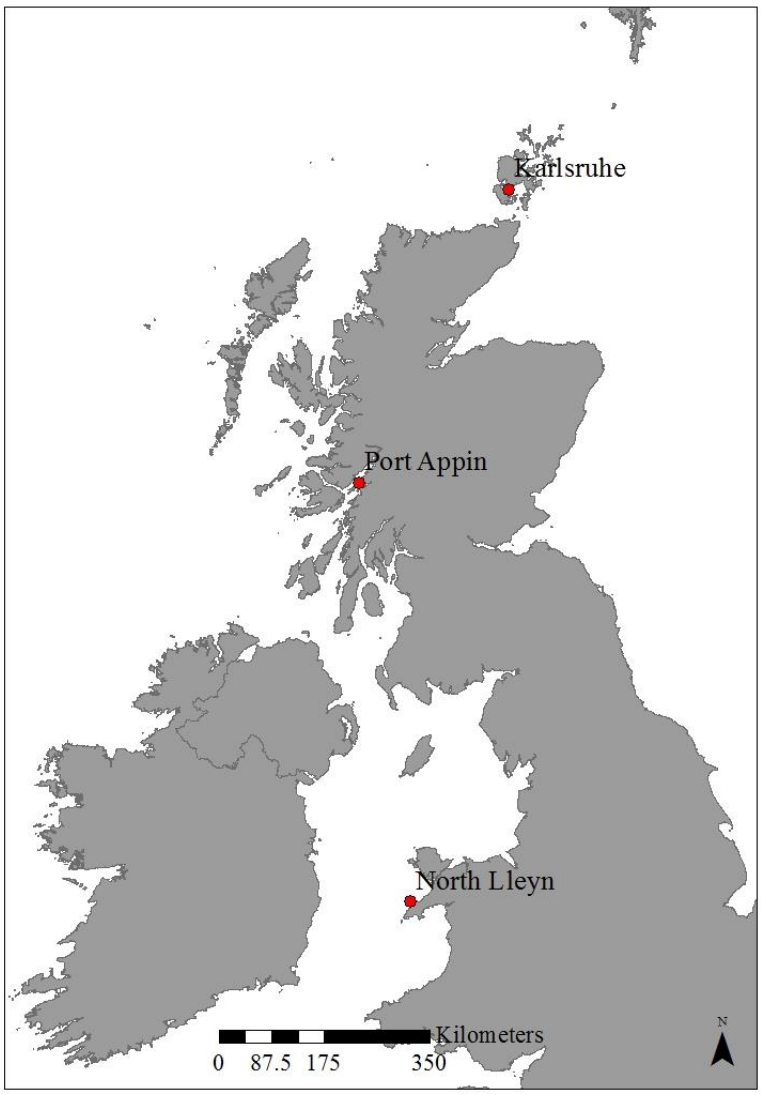


Figure 4.3. Locations of UK *M. modiolus* reefs (Karlsruhe, Port Appin, North Lleyn) used in thermal limits and oxidative stress experiments.

Experimental Set-up

Six 5L tanks were placed into each of two water baths (100L) maintained in a temperature controlled (TC) room at 10°C. Five animals from each population were haphazardly assigned to each of four experimental replicate tanks per population at 10°C (i.e. 5 animals tank^{-1} across 12 tanks; 4 tanks population^{-1}). The condition of each animal was checked before placement into tanks and any gaping animals were discarded. Bar heaters (Titanium 300KW heater, Aquamedic, Coalville, UK) were used to maintain and monitor experimental temperatures. A pump (Compact 600, Eheim Deizisau, Germany) was also placed in each water bath to ensure adequate circulation of seawater and maintain even heating across the treatment. Following, room and water temperatures were increased by

2°C per hour until 22°C at which point rate of increase was slowed to 1°C per hour until lethal temperature (i.e. LT50, where 50% of the population reached permanent gape) Temperature was monitored via a hand-held temperature meter (YSI Inc., Yellow Springs, USA) (Figure 4.4).

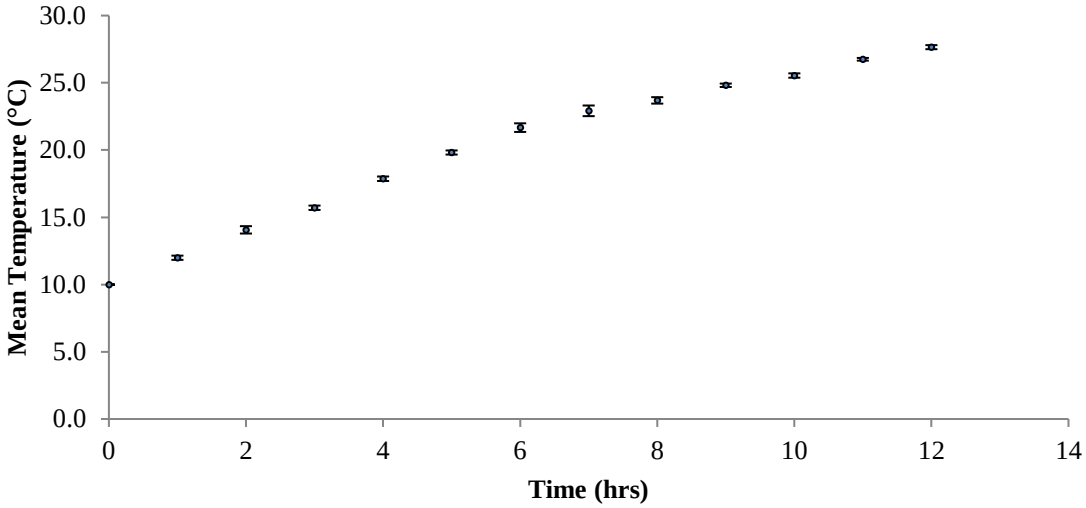


Figure 4.4. Mean temperature (±1SD) across replicate tanks during thermal limits experiment with Karlsruhe, Port Appin and North Lleyn populations.

Response to touch was assessed every hour and a score was assigned as per Table 4.2. A higher score indicated decreased response ability. A cumulative impact score for every replicate tank was calculated at each time point and used to determine a mean impact score per population per time point. Scores were plotted against temperature. Additionally, any spawning events were recorded.

Table 4.2. Scoring system for determining *M. modiolus* response to warming for determination of thermal limits.

Score	Observed response
0	shell valves closed
0	shell valves open, immediate closure when disrupted
1	shell valves open, delayed closure when disrupted
2	shell valves open, no closure when disrupted (continual gape)

Upon reaching LT50, heaters were turned off, the TC room temperature was set to 10°C, and animals were left overnight to recover. The following day, all surviving animals were returned to the main aquarium tanks (11°C, fully oxygenated) and post-experimental mortality (%) was assessed for two weeks on a weekly basis.

Data Analyses

Repeated measures general linear model (GLM) analyses (ANOVA) were used to examine changes in impact score over time. Additionally, one way ANOVAs were used to detect differences at specific temperatures. All data were tested for homogeneity of variances and normality to meet parametric assumptions. Where data failed to meet assumptions, square root or log10 transformations were applied.

Experiment 2. Effect of hypoxia to thermal limits in a *M. modiolus* reef population at the southern limit of the species' range

Animal Collection

Adult *M. modiolus* were collected by the Heriot-Watt Scientific Dive Team from the North Lleyn (Wales) reef (refer to p. 127, Figure 4.3) in May 2015 and transported to Heriot-Watt University. Animals were maintained in a flow-through closed circulation tank (300L) at approximately 11° and salinity 31-37ppt, and provided feed (mixed live phytoplankton) every two days. Air stones maintained oxygen levels at between 8-11 mg O_2 L^{-1}. Following three weeks' acclimation, animals were moved into experimental tanks.

Experimental Set-up

This part of the work was carried out with a MSc student, Matthew Smedley, as part of his thesis project (Smedley, 2015). Due to space and equipment constraints, the experiment was carried out in two stages on two separate days. The first stage examined thermal limits under normoxic conditions and was carried out as described above (refer to pp. 127-129) apart from the use of lidded 5L tanks in order to maintain oxygen conditions (Figure 4.5). Tanks were placed into each of two 100L water baths maintained at 10°C (via TC room). Thirty healthy (i.e. not gaping) mussels of a similar size (mean length=81.83mm ±SD 6.78) were moved from the acclimation tank to the tanks. Three animals were assigned per tank with five replicates per water treatment (i.e. ambient and warming). Temperatures and dissolved oxygen levels were monitored via a hand-held meter (YSI, Yellow Springs, USA) and temperature of the warming treatment was raised at a rate of 2°C per hour (Figure 4.6). Touch response of mussels was checked each hour and response was assessed as previously outlined (refer to p. 128, Table 4.2). Temperature was increased until LT50 (temperature at which 50% of animals reached "gape") was reached. Following, all animals received bubbled air (via airstones) and were left overnight to recover to 10°C. The following day, all surviving animals were returned to the main aquarium tank and post-experimental mortality was assessed for two weeks on a weekly basis.

Stage 2 of the experiment was carried out two days later with a new group of mussels from the same collection group to examine the effect of hypoxia to thermal limits. Conditions were identical to as previously described except that hypoxic seawater was

used across ambient and warming treatments (Figure 4.5). Hypoxia was achieved via bubbling of nitrogen gas into seawater until approximately 2mg 0_2 L^{-1} was achieved and maintained via sealing lids on all buckets. Hypoxia oxygen levels were set according to lethal levels for bivalves reported by Vaquer-Sunyer & Duarte (2008). Temperature increases and scoring were conducted as previously, until 50% of the mussels failed to respond to stimulus. Assessment was carried out quickly and with minimal disturbance to seawater, to aid in maintaining lowered oxygen concentrations. If necessary, extra nitrogen gas was bubbled into a tank to maintain hypoxic conditions. Temperatures and dissolved oxygen levels were monitored via a hand-held meter (YSI, Yellow Springs, USA) and temperature of the warming treatment was raised at a rate of 2°C per hour (Figure 4.6). Oxygen levels were maintained between 7.4-7.6 mg O_2 L^{-1} and 2.1-2.4 mg O_2 L^{-1} for normoxic and hypoxic treatments, respectively. Upon reaching LT50, all animals received bubbled air (via airstones) and were left overnight to recover to 10°C. The following day, all surviving animals were returned to the main aquarium tank and post-experimental mortality was assessed for two weeks on a weekly basis.

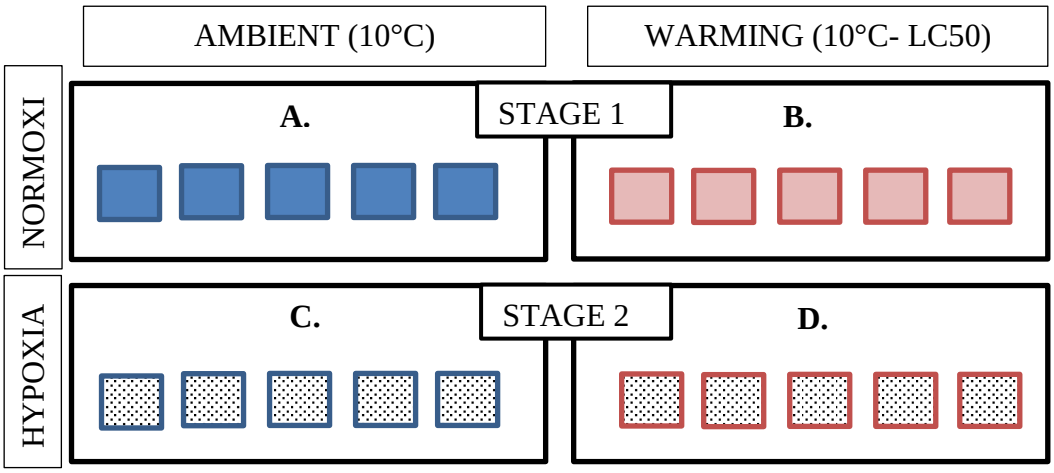


Figure 4.5. Experimental set-up for examining effects of hypoxia to thermal limits. Experiment was run in two parts (Stage 1 and Stage 2) in a multifactorial design to include four treatments: A. ambient temperature, normoxia; B: warming, normoxia; C: ambient temperature, hypoxia; D: warming, hypoxia. Blue (ambient) and red (warming) boxes with solid colour (normoxia) or dots (hypoxia) represent experimental replicate tanks (n=5 per treatment).

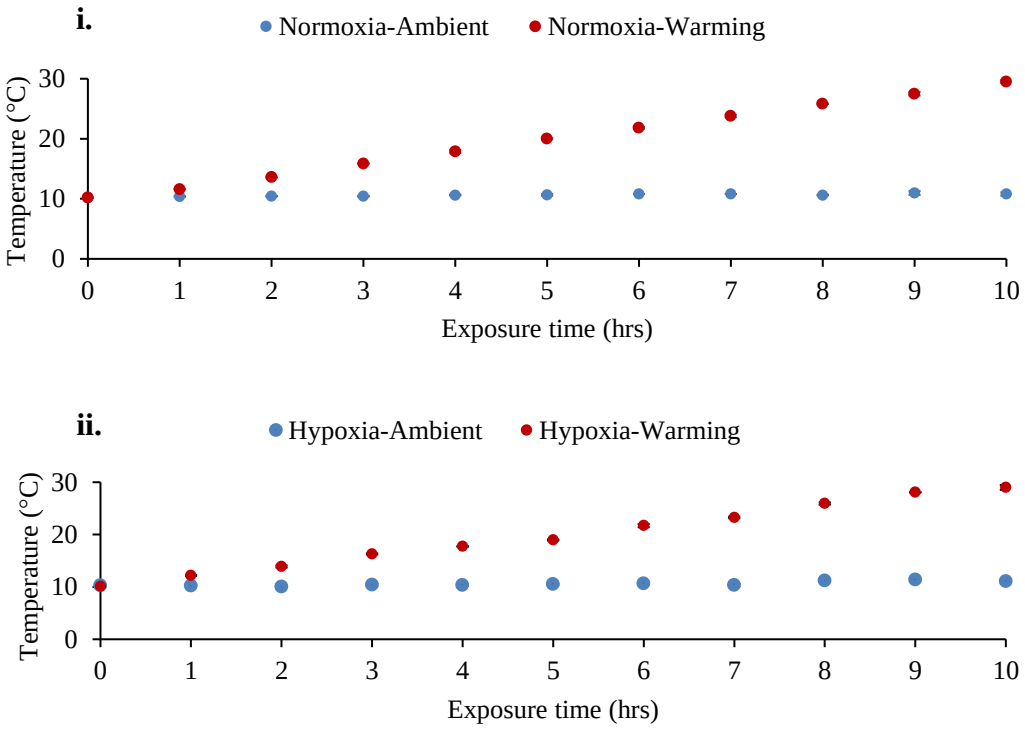


Figure 4.6. Temperature monitoring data for determining effects of hypoxia to thermal limits in *M. modiolus.* Means ±1SD are shown for (i) normoxic and (ii) hypoxic treatments.

Data Analyses

Repeated measures general linear model (GLM) (ANOVA) was used to examine changes in impact score over time. In addition, one-way ANOVAs were used to detect differences between normoxic and hypoxic groups at specific temperatures. All data were tested for homogeneity of variances and normality to meet parametric assumptions. Where data failed to meet assumptions, square root or log10 transformations were applied.

Experiment 3. Oxidative stress response in a *M. modiolus* reef population under acute warming conditions

Animal Collection

Adult *M. modiolus* were collected by the Heriot-Watt Scientific Dive Team from Karlsruhe (Scapa Flow, Orkney) in Spring 2014. Collection details are as previously outlined in Experiment 1 (refer to p. 126, Table 4.1). Animals were kept in flow-through aquarium tanks maintained at 15°C for a 4-week acclimation period prior to experimental start.

Experimental Set-up

Ten 12L tanks were placed into each of two water baths (100L) maintained in a TC room at 10°C. One water bath was equipped with bar heaters (with external control; hysteresis ±0.5°C) for raising temperature, hereafter referred to as the warming treatment (Figure 4.7). Each tank was filled with filtered seawater (10°C) from a re-circulated seawater supply and equipped with an airline and circulation pump. Eight *M. modiolus* were then haphazardly assigned to each tank. Animals were maintained overnight at ambient temperature of 9-10°C. The following morning, heaters were turned on in the warming treatment and seawater temperature was raised by 1°C hr^{-1} over 10 hours to a maximum of approximately 20°C (Figure 4.8). Temperature was monitored every hour with a handheld temperature meter (YSI, Yellow Spring, USA) and every minute via temperature loggers (Hobo Pendant Temperature Data Logger, Measurement Systems Ltd., Newbury, UK) placed in each water bath.

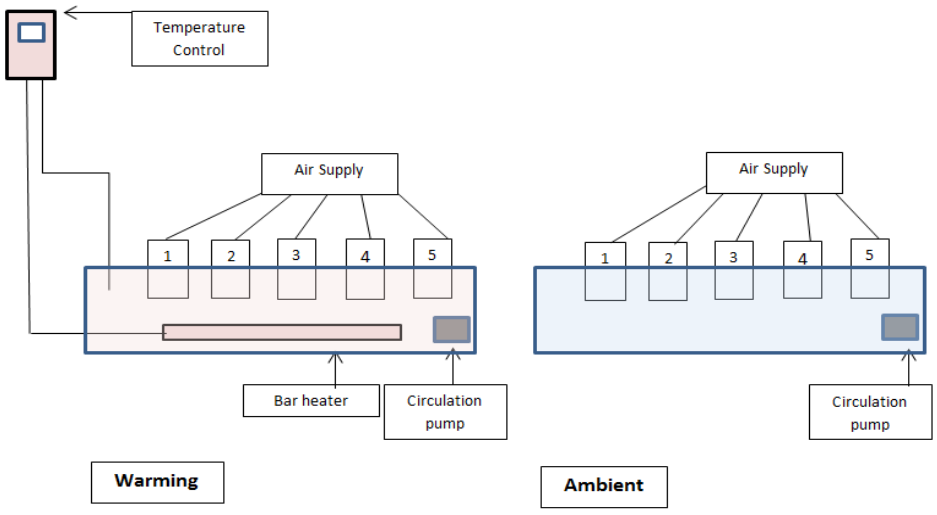


Figure 4.7. Experimental set-up for examining the effects of acute warming to oxidative stress response *in M. modiolus* (Karlsruhe population).

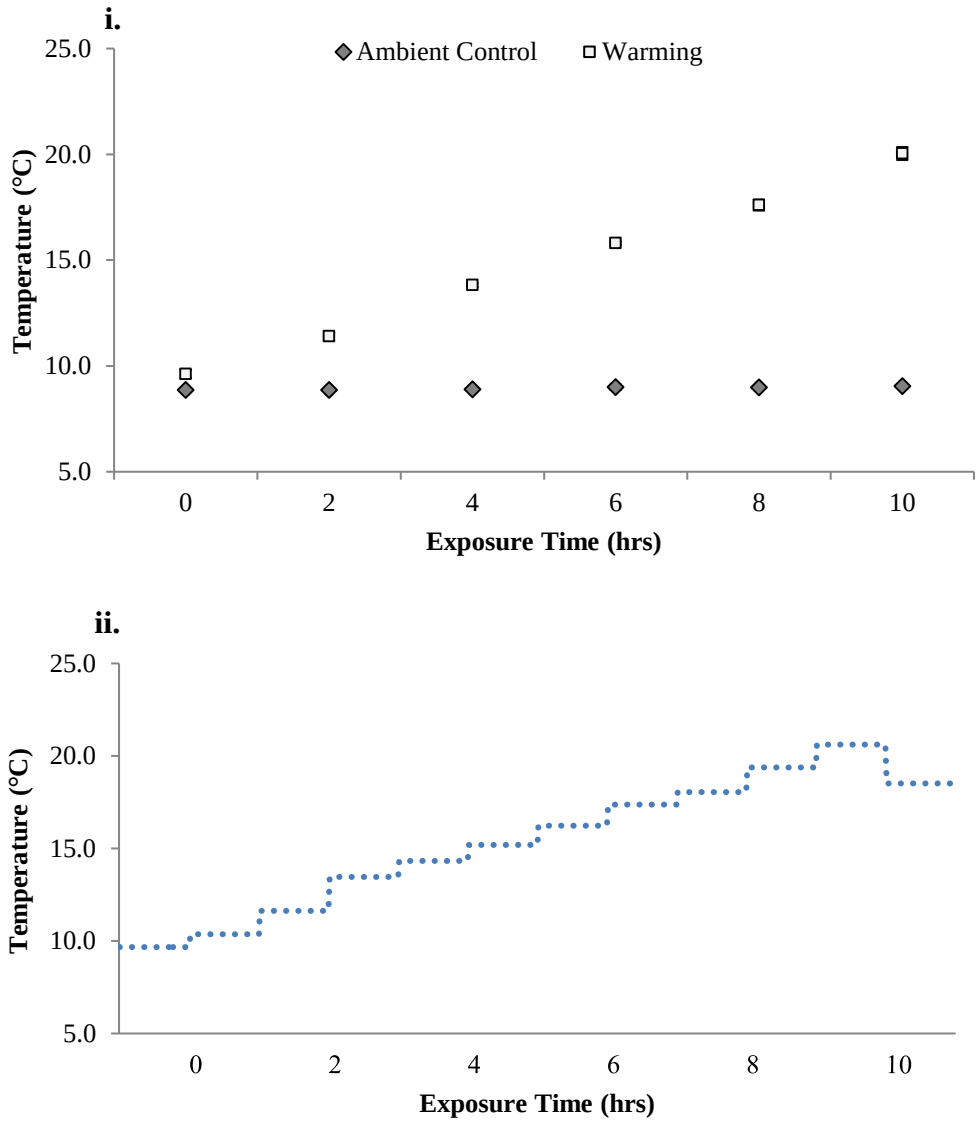


Figure 4.8. Experimental temperatures as measured with (i) hand-held temperature meter at the start of each sampling point and (ii) as measured with a temperature logger for the entire exposure (1 reading minute^{-1}) in warming treatment only.

Sampling

Oxidative stress biomarker values were measured at time=0 and every two hours subsequently. At each sampling time point, a single animal was removed from each experimental tank (n=5 per treatment). Following, the animal's shell was wedged open by approximately 5-10 mm via the byssal aperture allowing seawater to drain and exposing the posterior adductor mussel. Approximately 0.4-0.5 mL of haemolymph was drained into equal parts Hanks Balanced Salt Solution (HBSS) (adjusted with 22.2 g L^{-1} sodium chloride) from the posterior adductor mussel via insertion of a 21-gauge needle

on 1 mL syringe for later determination of cell viability and DNA damage via Comet assay. The needle was then removed and all sample was transferred to a 1.5 mL Eppendorf tube and kept on ice (Coughlan *et al*., 2002).

The posterior adductor mussel was then sheared to allow opening of the shell and dissection of both gills (left and right). The left gill was divided equally between two Eppendorf tubes, one with equal parts HBSS for later determination of DNA damage via Comet assay, and one with equal parts buffer solution (Tris-HCl 50mM, 0.15M KCl, pH 7.4) for later homogenization for determination of lipid peroxidation and protein concentration. Comet and cell viability samples were stored at 4°C and lipid peroxidation samples were stored at -20°C until subsequent analyses.

Oxidative Stress Biomarkers

Lipid Peroxidation

Preparation of Gill Tissue

Following sampling, gill tissue was homogenized within 24 hours. Frozen gill samples were homogenized on ice in 1:5 volumes of ice-cold buffer solution (Tris-HCl 50mM, 0.15M KCl, pH 7.4) for approximately 1 minute. The resulting homogenate was then equally divided between two 1.5 mL Eppendorf tubes before centrifugation at 13000 rpm for 12 seconds. The supernatant was removed without disturbing the pellet and frozen (-20°C) until determination of lipid peroxidation (via TBARS) and protein concentration.

TBARS and Protein Determination

Lipid peroxidation was determined as production of thiobarbituric acid reactive substances (TBARS) as per methods described by Camejo *et al.* (1998). A dilution series of 0, 2.5, 5, 15, 25 nMol 1,1,3,3-tetraethoxypropane (TEP*)* (>96%; Sigma-Aldrich) with ethanol was created in 15 mL centrifuge tubes. TEP concentrations were made up according to Table 4.3. 40 uL of each dilution series and all samples were added in triplicate to a 96-well plate (Fisher) (Figures 4.9 & 4.10). Next, 10 uL of BHT, 140 uL of PBS (with EDTA), 50 uL TCA and 75 uL TBA (in that order) were added to each set of triplicate wells (see Appendix A: Reagent Preparation). Samples were covered with the plate lid as each solution was added. Following, the plate was incubated at 55°C for 60 minutes.

In order to determine the amount of protein in each sample, a protein plate was also prepared. A BSA stock solution (1 mg mL^{-1}) was prepared with PBS (without EDTA). This stock solution was used to create a dilution series of 0, 0.2, 0.4, 0.4, 0.6, 0.8, 1.0 mg mL^{-1} with PBS (without EDTA). Samples were diluted 1:10 with PBS prior to addition to the well plate.

10uL of each dilution series and all diluted samples were added in triplicate to a 96-well plate (Fisher) (Figure 4.9 & 4.11). Next, 290 uL of Bradford's reagent were added to each set of triplicate wells. Following, the plate was incubated at 55°C for 5 minutes. Following incubation, UV-visible absorbances were read at 530 and 630 nm (TBARS) and 595 nm (protein) on a Spectrophotometer (Spectra Max). TBARS values were determined according to standard curve absorbance values, adjusted for dilution factor (x10), and expressed per mg protein.

Table 4.3. Standards curve dilutions for determination of TBARS. Varying volumes of TEP (1,1,3,3-tetraethoxypropane) and ethanol were mixed to create a standards curve of 0, 2.5, 5, 15, 25 nMol TEP.

Step	TEP (µl)		Ethanol (µl)	Conc (nMol)
0	0		2000	0
1	100 (of 1000000 uMol TEP)		9900	1,000,000
2	10 of 1	+	3090	25
3	600 of 2		336	15
4	400 of 3		800	5
5	400 of 4		400	2.5
6	300 of 5		1200	0.5

i.

TEP standards

	0	**0.5**	**2.5**	**5**	**15**	**25**		S1	S2	S3	S3	S5
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17

ii.

Protein standards curve

	0	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**		S1	S2	S3	S3	S5
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17

Figure 4.9. Plate layout for determination of (i) TBARS amounts and (ii) protein amounts in gill tissue. All samples (denoted S#) were run in triplicate.

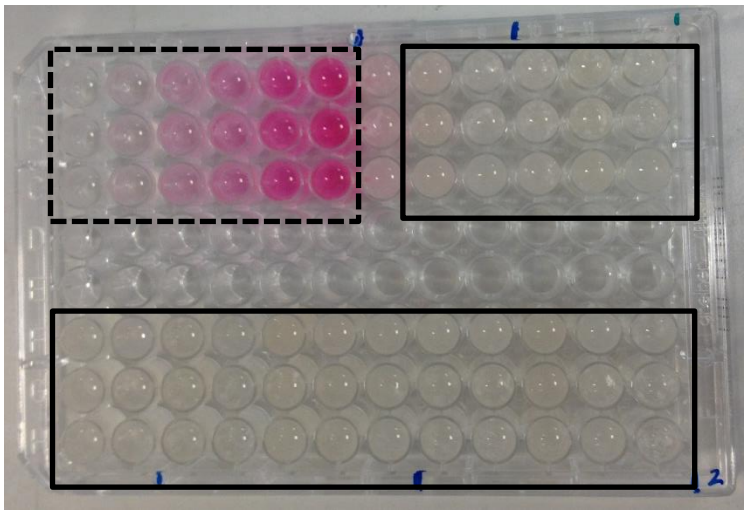


Figure 4.10. Example of TBARS well-plate for determination of lipid peroxidation. The standards curve (dashed line) was made up of 0, 2.5, 5, 15, 25 nMol 1,1,3,3-tetraethoxypropane (TEP). All samples (solid black line) were run in triplicate.

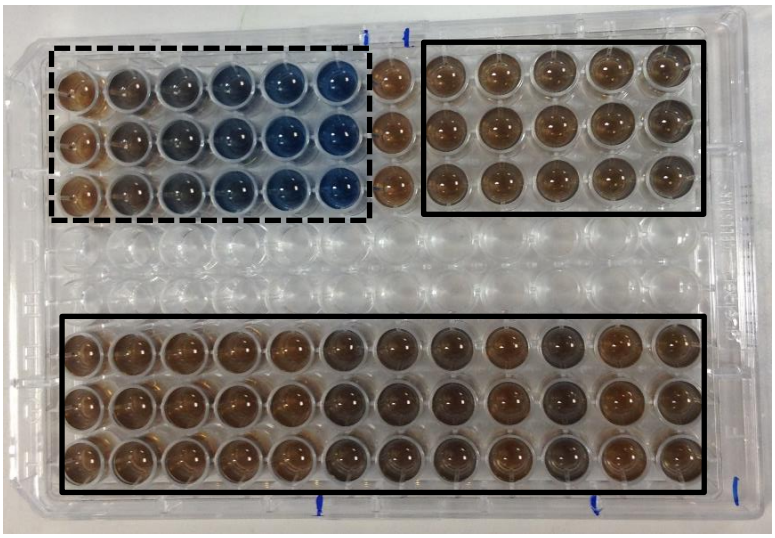


Figure 4.11. Example of well-plate for determination of protein values. The standards curve (dashed line) was made up of 0, 0.2, 0.4, 0.4, 0.6, 0.8, 1.0 mg mL^{-1} BSA solution (in PBS). All samples (solid black line) were run in triplicate.

DNA Damage

DNA damage was quantified via the Comet assay procedure, as adapted from Woods *et al.* (1999) and modified for mussels by Hartl *et al.* (2010). Please refer to Appendix A for specifics of reagent preparation.

Cell Viability

Cell viability (for assessing condition of haemolymph cells for Comet Assay) was assessed using the Trypan-Blue Exclusion method of Absolom [1986] using propidium iodide (PI) as a staining medium. 800 uL of HBSS (not adjusted with NaCl) and 200 uL of haemolymph sample were placed into a cytometer tube. A dilution (1:10) of PI stain was made up with distilled water. 20 µl of haemocyte suspension was mixed with 20 µl of the PI solution and pipetted onto a Neubauer Improved Haemocytometer plate covered with a cover slip (22 mm x 22 mm). A total of 100 cells were then counted with the number of stained (staining indicates unviable) and unstained cells (transparent indicates viable) noted. Counting was completed using a normal light optical microscope at 20x magnification. Cell viability was expressed as percentage viable (unstained) cells.

Isolation of Gill Tissue Cells

Each gill tissue sample was placed in a petri dish to which was added approximately 2.5 mL of HBSS adjusted with 22.2 g L^{-1} sodium chloride. Two clean scalpel blades applied in a scissoring motion were used to cut the gill tissue 20 times. All sample and HBSS was then transferred to a 15 mL centrifuge tube and kept on ice. 2.5 mL of a 1% Trypsin solution (2.5% Trypsin solution from porcine pancreas, SAFC, stored at -20°C) made up with HBSS was added to the sample. All samples were gyro-rocked for 10 minutes at 10 rpm at room temperature. Following, samples were filtered (40 um filter) in 15 mL tubes, topped up with HBSS to a 10 mL final volume and centrifuged for 10 minutes at 3000 rpm. Pellets were isolated into 1.5 mL Eppendorf tubes and resuspended in 0.5 mL HBSS.

Slide Preparation

Slides were prepared with a 1% Normal Gel Agarose (NGA) made up in Phosphate Buffered Saline (PBS). Briefly, approximately 100 uL of heated NGA was pipetted onto each microscope slide. While the gel was still liquid, another slide was used to drag the NGA across the entire slide. This provided a medium for the subsequent gel layers to build on. The slides were labelled with a pencilled dot to indicate the side prepared with

gel. The slide was then prepared with three layers, described as follows and illustrated in Figure 4.12.

1st layer: Slides were labelled and laid out on a hot plate held at approximately 45°C. 100 uL of heated NGA gel (kept in a 50°C water bath between heatings) was pipetted onto each slide and a cover slip was dropped at an angle onto the gel to form an initial layer. Slides were then moved to a cold room (4°C) for 20 minutes.

2nd layer: Slides were retrieved from the cold room. Cover slips were carefully removed from each slide by pushing gently with the thumb and sliding the cover off the gel layer. Slides were then laid onto the hot plate (45°C) with the cover slip propped on the slide edge. Haemolymph/gill cell samples were placed on ice. A 30 uL subsample of each sample was taken and deposited into a new 1. 5 mL Eppendorf tube. 70 uL of heated LMP gel (kept in a 50°C water bath between heatings) was added to the same tube before the entire amount (100 uL) was quickly pipetted up and onto the slide and cover slip dropped at an angle onto the liquid gel mixture. Following completion of all samples, the slides were placed in a cold room (4°C) for 20 minutes.

3rd layer: Slides were retrieved from the cold room. Cover slips were carefully removed from each slide by pushing gently with the thumb and sliding the cover off the gel layer. Slides were then laid onto the hot plate (45°C) with the cover slip propped on the slide edge. A new batch of LMP gel was heated up and kept in a 50°C water bath between heatings. 105 uL of LMP gel was added to each slide and covered with cover slip. All slides were placed in the cold room (4°C) for 20 minutes.

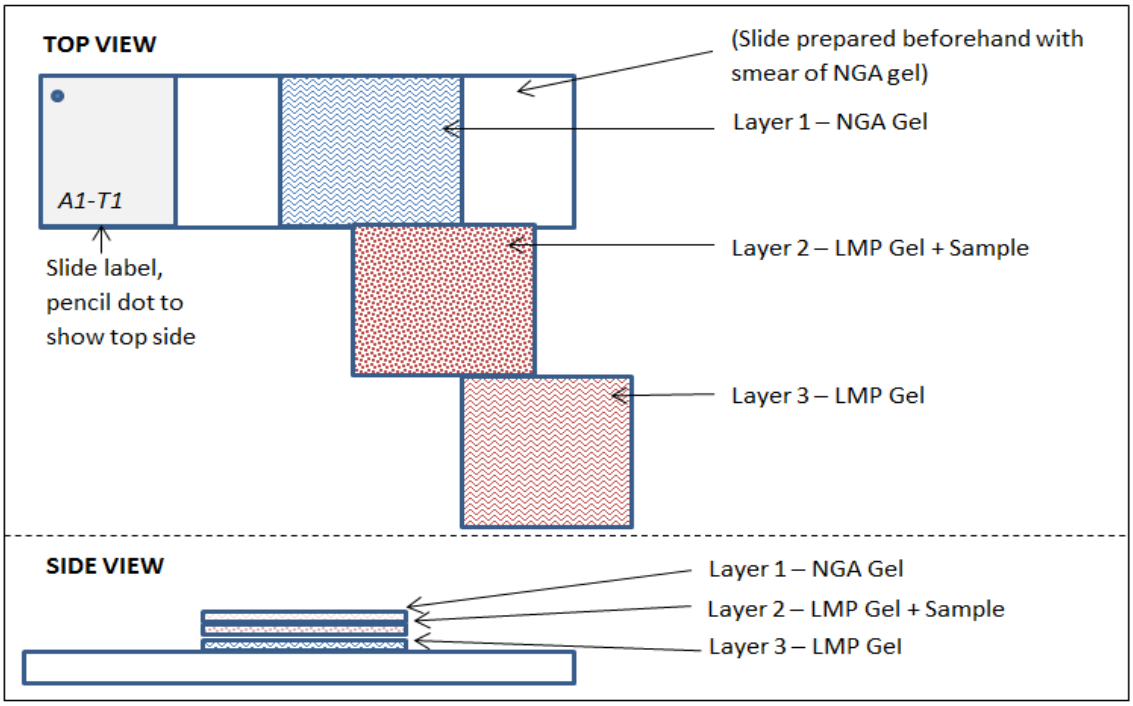


Figure 4.12. Comet assay slide preparation showing three layers of gel from top and side views. NGA=normal gel agarose; LMP=low melting point agarose.

Cell Lysis

Following the final cooling period, cover slips were removed and all slides were placed into lysis solution. Lysis solution was prepared 24 hours beforehand and stored at 4°C. Slides were left in lysis solution for 1-7 days before gel electrophoresis.

Gel Electrophoresis

An electrophoresis tank was set-up in a cold room (4°C). Up to 40 slides were taken from lysis solution and arranged in the tank (5 columns of 8 slides). Extra blank slides were used at the bottom of each column to wedge slides into tank and prevent movement. Electrophoresis buffer solution was slowly added to the tank until all slides were just covered. The lid of the tank was replaced and slides were left for 30 minutes in the dark. This period of time allowed the DNA to unravel.

Following 30 minutes, an ammeter was attached to tank electrodes (positive and negative) and turned on. Additionaly buffer solution was added to the tank via a small funnel until the ammeter read 300 mA. The slides were left to electrophorese for 25 minutes in the dark. Following 25 minutes, the ammeter was turned off and all slides were removed

onto paper towel. Slides were tripled cleaned with TRIS buffer with a five-minute waiting period between cleanings. Following the final cleaning and waiting period, slides were washed with distilled water and all excess liquid was tipped off slides. All slides were then stained with Gel Red (0.02% solution in distilled water) and left for five minutes before excess was tipped off and cover slip applied. All slides were placed on damp paper towel before taken to the microscope for scoring.

Scoring

Fifty randomly selected nucleoids per slide were analysed under green fluorescence at 40x magnification using a Zeiss Axiophot epifluorescence microscope equipped with Zeiss AxioCam MRm digital camera (200x magnification). Cells were scored using the COMET ASSAY IV image analysis software package (Perception Industries). Five slides were scored for each treatment group. Estimates of DNA strand breakage were expressed as the percentage of DNA found in the comet tail (Collins, 2004). Figure 4.13 demonstrates how the software determined DNA damage.

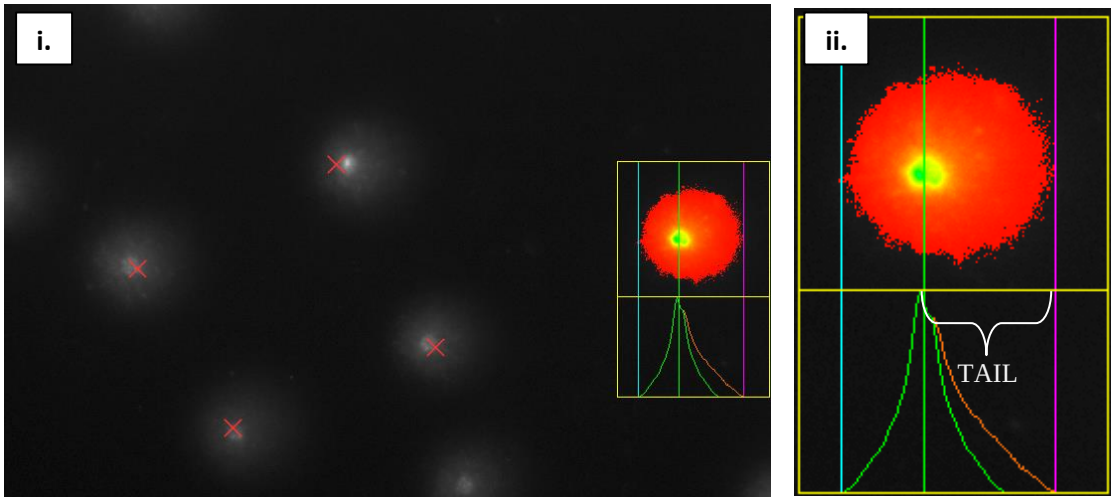


Figure 4.13. (i) *M. modiolus* gill cells as visualised with a Zeiss Axiophot epifluorescence microscope for determination of DNA damage via the comet assay. A red "x" indicates that a cell has been scored. Image to right (ii) illustrates how each cell is scored. The proportion of DNA that has moved via electrophoresis into the tail of the cell (labelled TAIL) determines cell damage (i.e. strand breaks).

Data Analyses

1-way ANOVA with post hoc were applied to detect differences in DNA damage in gill tissue and haemolymph and lipid peroxidation in gill tissue. All data were tested for homogeneity of variances and normality to meet parametric assumptions. Where data failed to meet assumptions, square root or log10 transformations were applied.

Experiment 4. Comparison of oxidative stress response in *M. modiolus* reef populations under short-term exposure to site-specific warming conditions

Animal Collection

Experiments were run using the same populations (North Lleyn, Port Appin and Karlsruhe) as were collected for determination of thermal limits (refer to pp. 126-127 for collection details).

Experiment Set-up

Three separate experiments were carried out with each of the three populations at temperature conditions representative of each population's current (i.e. collection temperature) and future (i.e. collection temperature + 5°C) temperature conditions (Table 4.4). For each experiment, fifty animals from the North Lleyn, Port Appin or Karlsruhe population were haphazardly assigned to ten 12 L tanks (n=5 per tank) in a temperature controlled room (kept at the control temperature for that population). Each tank was filled with filtered seawater from a re-circulated seawater supply and equipped with an airline. Five tanks were assigned to each of two water baths (refer to p. 133, Figure 4.7 for diagram of set-up). Initially, water baths were maintained at collection temperature for the population (Table 4.4). Animals were then left for 4 hours to acclimate to tanks prior to baseline sampling (n=10). Following, one water bath was warmed by 5°C over 5 hours (1°C/hr) to represent potential future climate change conditions (for that population) whilst the other was maintained as the control temperature (Table 4.4). Warming was achieved via the use of submerged bar heaters (Aquamedic, 500 KW) and circulation pumps. Pumps also ensured circulation of water to maintain constant temperature across replicate tanks.

Table 4.4. Collection and future temperatures for Karlsruhe, Port Appin and North Lleyn *M. modiolus* populations

Population	**Collection Temperature (°C)***	**Future Climate Change Temperature (°C)****
Karlsruhe (Orkney Islands)	8	13
Port Appin (Scotland)	11	16
North Lleyn (Wales)	14	19

*temperature of control group; **temperature of warming group

Daily water changes (2/3 volume) were carried out for all tanks. During water changes, animals were left submerged in bottom of tanks. Following each water change, 5 L water canisters were re-filled with seawater and placed in water baths to ensure correct seawater temperatures for the subsequent water change. A 12:12 photoperiod was applied.

Sampling

Following seven days of exposure, one animal from each tank was dissected for determination of DNA damage (via Comet assay), lipid peroxidation (via TBARS assay) and antioxidant production (SOD). Comet and TBARS samples were taken as previously described (refer to pp. 134-135). The TBARS samples were also used for later SOD determination.

Oxidative Stress Biomarkers

DNA damage

DNA damage was determined via the Comet assay as previously described (refer to pp. 139-142.

Lipid Peroxidation

Preparation of gills cells was carried out as previously described (refer to p. 133). Lipid peroxidation was determined via the TBARS assay as previously described (refer to pp. 135-138).

Superoxide Dismutase

Supernatant from remaining gill homogenate as prepared for lipid peroxidation (refer to p. 135) was centrifuged at 40 000 g for 60 minutes at 4°C to obtain the cytosolic fraction which was removed into a new tube and frozen (-80°C) for later determination of superoxide dismutase (SOD). SOD levels were determined with a SOD Assay Kit (19160, Sigma, etc.). 20uL of sample were added in triplicate to a 96-well plate. Three blanks (Blanks 1-3) were included using 20 uL distilled water (Blanks 1 and 3) or 20 uL sample solution (Blank 2). 200uL of WST working solution (refer to Appendix A for details of reagent preparation) was then added to all wells and all wells were mixed. 20 uL of dilution buffer was added to Blanks 2 and 3. Finally, all samples and Blank 1 received 20 uL of enzyme solution (refer to Appendix A) and were mixed well. The plate was incubated for 20 minutes at 30°C before absorbances were read at 450 nm on the

microplate reader. SOD activity (inhibition rate %) was determined as: SOD activity = (((Abs blank1 – Abs blank3) – (Abs sample-Abs blank2)) / (Abs blank 1 – Abs blank3)) * 100 as per the Sigma SOD protocol.

Data Analyses

1-way ANOVAs were carried out to determine the effect of warming to DNA damage, and lipid peroxidation levels within populations (i.e. compare baseline, control and warming groups) and between populations. All data were tested for homogeneity of variances and normality to meet parametric assumptions. Where data failed to meet assumptions, square root or log10 transformations were applied. Significance level $p<0.05$.

Experiment 5. Oxidative stress response in a *M. modiolus* reef population under short-term exposure to hypoxia

Animal Collection

Adult *M. modiolus* (n=100) were collected by dredge tow (between 52.24.050, 4.20.653 and 54.24.417, 04.20.643) from Ramsey Bay, Isle of Man by the Isle of Man Government Fisheries Group in September 2014. Animals were placed in cold boxes with seawater and ice packs (temperature ~ 8°C) and transported to Heriot-Watt University within 24 hours of collection. Seawater oxygen levels were maintained via use of portable air lines with air stones and mortality rates were <1%. Animals were transferred to a flow-through aquarium tanks filled with re-circulated seawater (approx. 12°C) and fed twice weekly with algae (water dosed with 5L of 1:1 *T-iso lutea* and *Tetraselmis suecica*). Animals were maintained at these conditions for three months prior to experimental start.

Experimental Set-up

Five 5L lidded buckets were placed into each of two 100L water baths maintained at approximately 12°C (via TC room). 5 buckets were randomly selected to receive hypoxic water. Hypoxic water was created via bubbling of nitrogen gas (BOC) through an air stone until oxygen levels reached approximately 2mg/L. Levels were maintained with sealed lids on all buckets. 50 healthy (i.e. not gaping) mussels of a similar size (mean length=107.4mm ±SD 5.18) were moved from the acclimation tank to the buckets (5 per bucket). Temperature was maintained at 11.49±0.25°C for the duration of the experiment. Daily water changes were made. Nitrogen gas was bubbled into large canisters (~20L) of seawater (previously stored in the TC room overnight to ensure correct temperature) until an oxygen level of approximately 2mg L^{-1} was reached. Water changes were then made as quickly as possible to all hypoxic treatment buckets. Water was poured slowly and smoothly to reduce aeration. Following, all oxygen levels were measured and if necessary, buckets received extra bubbling of nitrogen gas until desired oxygen levels were reached. All normoxic treatment buckets also received a water change at this point with normoxic water (previously stored in large canister in the TC room overnight to ensure correct temperature). Oxygen levels were checked and recorded for all buckets before and after water changes (Figure 4.14). The experiment was carried out for seven days, following which 1 animal from every bucket was removed and gill tissue dissected and flash-frozen in liquid nitrogen for subsequent determination of SOD, TBARS and protein carbonyl levels (referred to as post-exposure measures). All

remaining animals were then exposed to fresh normoxic seawater for a 6-hour recovery period. An additional animal was then removed from every bucket and gill tissue dissected and flash-frozen in liquid nitrogen for subsequent determination of SOD, TBARS and protein carbonyl levels (referred to as post-recovery measures).

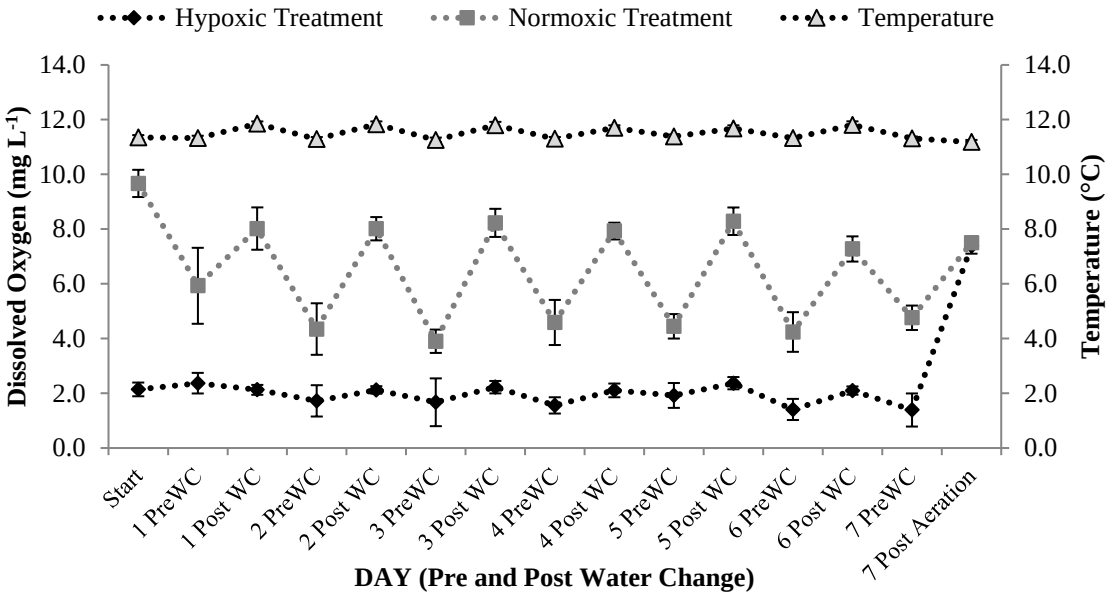


Figure 4.14. Dissolved oxygen and temperature conditions in hypoxic and normoxic treatments for determining the impact of hypoxia to oxidative stress response in Ramsey Bay (IOM) *M. modiolus* reef population. PreWC=before water change; PostWC=after water change.

Oxidative Stress Biomarkers

Lipid Peroxidation

Preparation of gills cells was carried out as previously described (refer to p. 132). Lipid peroxidation was determined via the TBARS assay as previously described (refer to pp. 135-138).

Superoxide Dismutase

SOD was determined via the SOD assay (Sigma, 2016) as previously described (refer to pp. 145-146).

Protein Carbonylation

A Protein Carbonyl Content Assay Kit (Catalog Number MAK094, Sigma Aldrich) was used to determined protein carbonyl levels in gill samples. Supernatant was obtained from the same sample used for TBARS measurements (see Section). 100 uL of supernatant was placed in a 1.5 mL tube to which 10 uL of 10% Streptozocin Solution (provided in kit). The solution was then incubated at room temperature for 15 minutes before centrifugation at 12000g for 5 minutes. 100uL of the resulting supernatant was transferred to a new 1.5 mL tube to which 100uL of 2,4-dinitrophenylhydrazine (DNPH, provided in kit) was added. The sample was vortexed and then incubated for 10 minutes at room temperature. 30 uL of 100% trichloroacetic acid (TCA) solution was then added and the sample was then vortexed before incubation on ice for five minutes. Next, the sample was centrifuged at 13000 rpm for 2 minutes. The supernatant was removed to leave a pellet to which 500 uL of ice cold acetone was added. The sample was then placed in a sonication bath for thirty seconds before incubation at -20°C for five minutes. Following the sample was centrifuged at 130000 g for 2 minutes. Acetone was carefully removed (so as not to disturb the pellet) and discarded, and 200 uL of 6M guanidine was added to the pellet. The sample was sonicated for 5 seconds. Finally, 100 uL of the sample was added to a 96 well plate and absorbance was read at 375 nM. Protein carbonyl content was determined according to the Sigma protocol (2016): Carbonyl content = $Absorbance_{375}$/6.364*100 where 6.364 = millimolar extinction coefficient (εmM= 22 $mM^{-1}cm^{-1}$) * pathlength in a well for enclosed 96-well plate (0.2893 cm); and 100=total volume in well (μL). Sample protein concentrations determined for TBARS calculations were used to present results in nmol protein carbonyl per mg protein.

Data Analyses

1-way ANOVA were applied to test for differences in TBARS, SOD and protein carbonyl between baseline, post-exposure and post-recovery groups, and to compare the effect of hypoxia within post-exposure and post-recovery groups. All data were tested for homogeneity of variances and normality to meet parametric assumptions. Where data failed to meet assumptions, square root or log10 transformations were applied. Significance level was $p<0.05$.

Experiment 6. Comparison of oxidative stress response in northern and southern *M. modiolus* reef populations under medium-term exposure to warming and hypoxia conditions

Animal Collection

Approximately 100 adult *M. modiolus* were collected sub tidally by the Heriot-Watt Scientific Dive Team from the Karlsruhe reef (Orkney, Scotland) (58.891, -3.188; 25m) in April 2015 and from the North Lleyn reef (Wales) (52.936, -4.65; 25m) in May 2015. Mean shell lengths of Karlsruhe and North Lleyn *M. modiolus* were 108.3mm (±SD10.0mm) and 87.5mm (±SD7.9mm), respectively. Animals were placed in cold boxes with seawater and ice packs (temperature ~ 8°C) and transported to Heriot-Watt University within 24 hours of collection. Animals were transferred to a flow-through aquarium tanks (300L) filled with fully oxygenated re-circulated seawater (11°C, ~8mg O_2/L)). Mortality rates following transport were <1%.

Acclimation

Animals were cleaned of all shell epifauna and left to acclimate for approximately 4-6 weeks. Acclimation conditions are provided in Table 4.5 Animals were provided feed (5L of 1:1 *T-iso lutea* and *Tetraselmis suecica*) twice weekly. Conditions were regularly checked and any dead mussels removed. Mortality rates during acclimation were <5%.

Table 4.5. Acclimation temperature, salinity and dissolved oxygen values for Wales and Orkney populations

Population	Temperature (°C)	Salinity (ppt)	Dissolved Oxygen (mg O_2 L^{-1})
Karlsruhe (Orkney Islands)	11.58 (±0.46)	34 (±2.45)	9.24 (±.95)
North Lleyn (Wales)	12.21 (±0.78)	36.9 (±0.3)	8.54 (±0.66)

Experimental Set-up

Following the acclimation period, mussels were transported to St. Abbs Marine Station (approximately 1 hour transport time) where they were haphazardly divided into three temperature controlled water baths pre-set to 14°C. 10 five litre tanks were placed into each water bath and four mussels were placed into each tank. 10°C and 18°C treatment

tanks were acclimated over three days from 14-10°C or 14-18°C, respectively, at 2°C per day, to create three temperature treatments (10°C, 14°C, 18°C). Temperatures were chosen to cover the normal temperatures experienced by *M. modiolus* in the field (Kent, 2015) and approximately upper thermal limits (following Halanych *et al*., 2013). All experimental tanks received a constant supply of air via air stones and fresh seawater from the adjacent ocean areas at St. Abbs via a central delivery line dividing into each tank (Figures 4.15 & 4.16). Water supply to each tank was controlled via a pressure regulation system into the bottom of each tank and tanks overflowed to waste. Temperature was controlled via bar heaters (Titanium 300KW heater, Aquamedic, Coalville, UK) and combined heating/chilling refrigeration units (Commercial chiller/heater TC500, TECO, Ravenna, Italy). Light/ dark periods followed natural levels with the transparent design of the aquarium building allowing diffusion of natural light into the tanks. This was moderated via the use of covers to provide the lower levels of light expected in *M. modiolus* habitats. Mussels were maintained in these conditions for 21 days with temperature recorded daily with a hand-held temperature meter (YSI ProDSS, YSI, Yellow Spring, USA) (Table 4.6) and monitored via in-tank temperature loggers (Hobo Pendant Temperature Data Logger, Measurement Systems Ltd., Newbury, UK)). Tanks were cleaned once per week via siphoning (50% water volume) to remove fecal material.

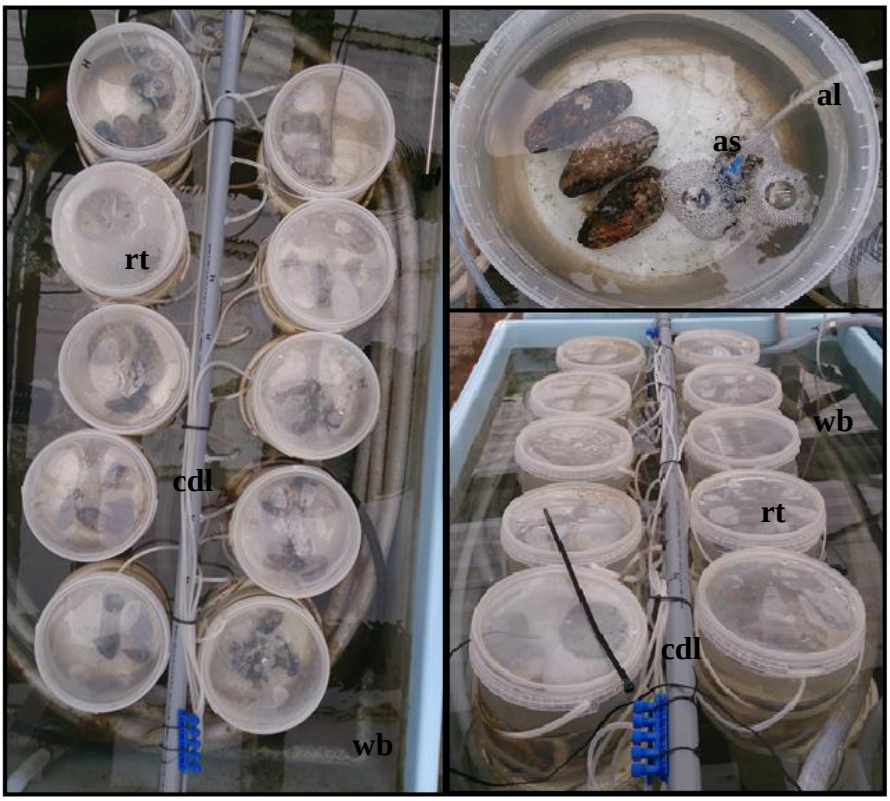


Figure 4.15. Experimental set-up for investigation of warming and hypoxia to oxidative stress response in *M. modiolus*. 10 replicate tanks (rt) within a heated water bath (wb) received seawater supply from a central delivery line (cdl) and air supply via individual airlines (al) and airstones (as).

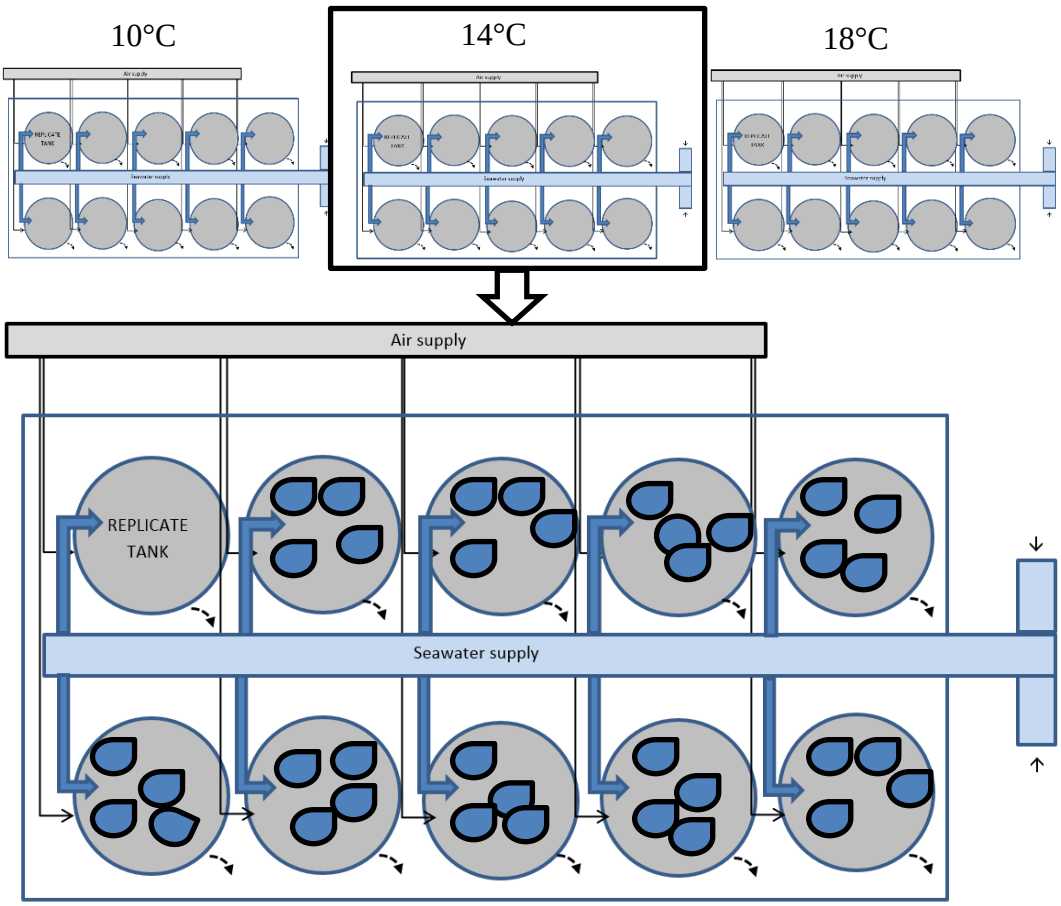


Figure 4.16. Experimental set-up for investigation of warming and hypoxia to oxidative stress response. *M. modiolus* were exposed to (i) one of three temperature treatments (10°C, 14°C, 18°C). Lower diagram (ii) shows detail of temperature treatment, consisting of 10 replicate tanks (4 mussels $tank^{-1}$). Water delivery was via a central pipe line (pl) and overflowed to waste. All tanks received a constant supply of air from a central airline supply via airstones.

After 21 days, water supply was turned off and animals were held at either normoxic (n=5) or hypoxic (n=5) conditions for one week under the same temperature treatment as the previous three weeks. Animals were assigned to either oxygen treatment randomly (Figure 4.17) and received daily water changes with either normoxic (~8mg O_2 per L) or hypoxic (~2mg O_2 per L) seawater. Prior to delivery to tanks, seawater was pre-warmed/cooled via water baths and hypoxic water was created via bubbling of nitrogen gas (BOC) through an air stone until oxygen levels reached approximately 2mg L^{-1} as per levels representative of hypoxia for bivalves reported by Vaquer-Sunyer & Duarte (2008).

Oxygen levels were maintained with sealed lids on all tanks and monitored twice daily (Table 4.6).

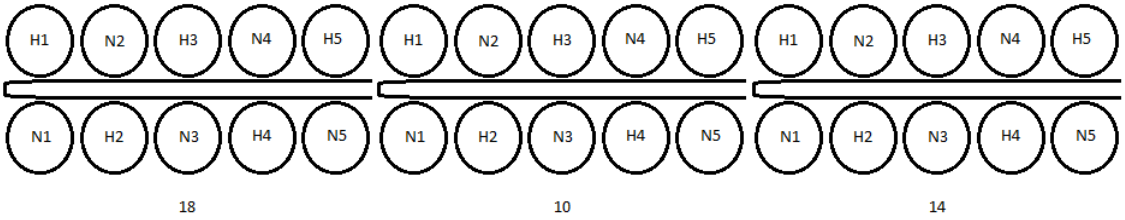


Figure 4.17. Assignment of hypoxia (H1-H5) and normoxia (N1-N5) replicates for each temperature treatments (10°C, 14°C, 18°C) (shown in order as per actual aquarium set-up).

Table 4.6. Mean temperatures and mean oxygen concentrations across experimental treatments for North Lleyn and Karlsruhe *M. modiolus* populations. Standard deviations are provided in parentheses.

Population	**Factor**	**Unit**	**Treatment**					
			10°C		**14°C**		**18°C**	
			N	**H**	**N**	**H**	**N**	**H**
North Lleyn	Temperature	°C	10.29 (0.84)		13.96 (1.14)		17.75 (0.50)	
	Oxygen concentration	mg O_2 L^{-1}	7.60 (0.20)	3.08 (0.34)	7.12 (0.25)	2.83 (0.20)	5.99 (0.78)	2.71 (0.21)
Karlsruhe	Temperature	°C	10.41 (0.61)		14.23 (0.63)		17.51 (0.74)	
	Oxygen concentration	mg O_2 L^{-1}	8.79 (0.17)	3.66 (0.38)	8.14 (0.20)	3.05 (0.14)	7.77 (0.09)	2.64 (0.49)

N=normoxic, H=hypoxic

Due to the loss of comet assay gill samples from the 10°C, 14°C and 18°C temperature treatments (normoxic conditions only) during laboratory processing, a second experiment was carried out to replace lost samples. Experimental animals were from the same population and collection event. The experiment was run at Heriot-Watt University in a static aquarium system where animals underwent a 28-day temperature exposure at 10°C, 14°C and 18°C at normoxic oxygen levels (approximately 8mg DO L^{-1}). Temperatures were achieved via the use of a temperature controlled room (10°C) and water baths equipped with bar heaters (14°C, 18°C) and water pumps to ensure even temperature

mixing. Mean temperatures (±1SD) for the repeat temperature experiment were 10.29(±0.02)°C, 13.62(±0.38)°C and 18.5(±1.53)°C. Animals received 2/3 tank volume water changes every other day at which time they were fed Shellfish Diet (Reed Mariculture). Feed amount was calculated as 3.6 mL of Shellfish diet per 100g meat wet weight as per Reed Mariculture guidelines.

Sampling

Following one week at combined temperature-oxygen conditions, one animal was removed from each tank. Haemolymph and gill samples were taken (as described previously; refer to pp. 131-132) and preserved in HBSS (adjusted) for determination of DNA damage via Comet Assay. Additionally, gill samples were taken for determination of TBARS, SOD, catalase and protein carbonylation. Samples were frozen in liquid nitrogen, transported back to HWU on dry ice, and stored at -70°C until subsequent processing. Identical experimental procedures were carried out for Orkney animals (in March-April 2015) and Wales animals (June 2015).

Oxidative Stress Biomarkers

DNA damage

Isolation of gill cells and determination of DNA damage via the Comet assay was carried out as previously described (refer to pp. 139-142). Samples from the original experiment were processed by HWU student Matthew Smedley as part of his MSc project (Smedley, 2015), under supervision of the author and Dr. Mark Hartl. The author completed later processing to replace samples that were lost (i.e. North Lleyn normoxic samples).

Lipid peroxidation

Preparation of gills cells was carried out as previously described (refer to p. 135). Lipid peroxidation was determined via the TBARS assay as previously described (refer to pp. 136-139).

SOD

SOD was determined via the SOD assay (Sigma, 2016) as previously described (refer to pp. 145-146).

Protein Carbonylation

Protein carbonylation was determined via the protein carbonyl assay as previously described (refer to p. 149).

Catalase

Catalase levels were determined according to a Sigma Aldrich assay kit (CAT100) via the colorimetric assay reaction. All solutions were prepared as outlined in Appendix A. All solutions were prepared ahead of time except for the catalase control and H_2O_2 standard which were made up fresh on the day of readings. A standards curve of 0, 12.5, 25, 50 and 75 nMol H_2O_2 was made up with 1x assay buffer as per the Sigma protocol (Sigma, 2016). 1 mL of colour reagent was added to a 10uL aliquot of each dilution. The solutions were left for 15 minutes and following, absorbance was read at 520 nm. Absorbance values and H_2O_2 concentrations were plotted to create a standards curve.

Next, 5 uL of the cystolic fraction of each prepared gill sample was pipetted into a sterile Eppendorf tubes (1.5 mL) and placed on ice. 70 uL of 1x assay buffer was then added to each sample. Additionally, 75 uL of 1x assay buffer was added to a separate tube to act as a control. 25 uL of Colorimetric assay substrate solution (200 mM H_2O_2) was added to all tubes to start the reaction. All tubes were inverted to mix and left for 3 minutes. Following, 900 uL of stop solution was added to all tubes and all tubes were inverted to mix. 10 uL of each sample was then removed and placed into a new tube. 1 mL of colour reagent working solution was added and mixed by inversion. All tubes were then incubated at room temperature for 15 minutes and absorbance was measured at 520 nm using glass cuvettes.

The amount of H_2O_2 in each sample was determined via the standards curve and adjusted for the control value. Catalase activity was then determined as: (umoles H_2O_2 x 100)/(v*t) where 100=dilution factor, v=sample volume, and t=catalase reaction duration. Catalase values were expressed in catalase units (U) where one unit equals the amount that will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM hydrogen peroxide (Sigma, 2016). Final catalase units were expressed per mg protein as per protein values determined for lipid peroxidation.

Analyses

Data were tested for normality and homogeneity of variances (Levene's Test). If necessary, data were log-transformed to meet assumptions. Two-way ANOVAs were carried out to determine the effects of temperature and oxygen content (and interaction). Data are presented as means ±1SE unless otherwise stated. Statistical analyses were performed using SPSS software (SPSS 14, SPSS INC, Chicago, IL, USA). All reported significance levels are $p<0.05$.

4.5 Results

Experiment 1. Comparison of thermal limits in *M. modiolus* reef populations from across a latitudinal gradient

Decreased response ability was first detected at 22°C, 24°C and 25°C and LT50 values determined to be 27°C, 28°C and 28°C in Orkney, Wales and Appin populations, respectively. Mussels from the Appin population maintained the lowest impact score at LT50. Repeated measures GLM detected no difference in impact scores between populations over time ($F_{(2,10)}=2.049$, $p=0.185$). One-way ANOVA detected a near significant difference ($F_{(2,10)} =3.972$, $p=0.058$) in impact score between the Karlsruhe population and North Lleyn at 27°C (Figure 4.18). Spawning was induced in 50% of North Lleyn tanks at 20°C, 25% of Karlsruhe tanks at 22°C, and 0% of Port Appin tanks. There was 100% survival across all populations following 24-hour recovery at 15°C. Following a two-week recovery period, the Wales population experienced the highest mortality (50%) followed by Appin (40%) and Orkney (30%) populations (Figure 4.19).

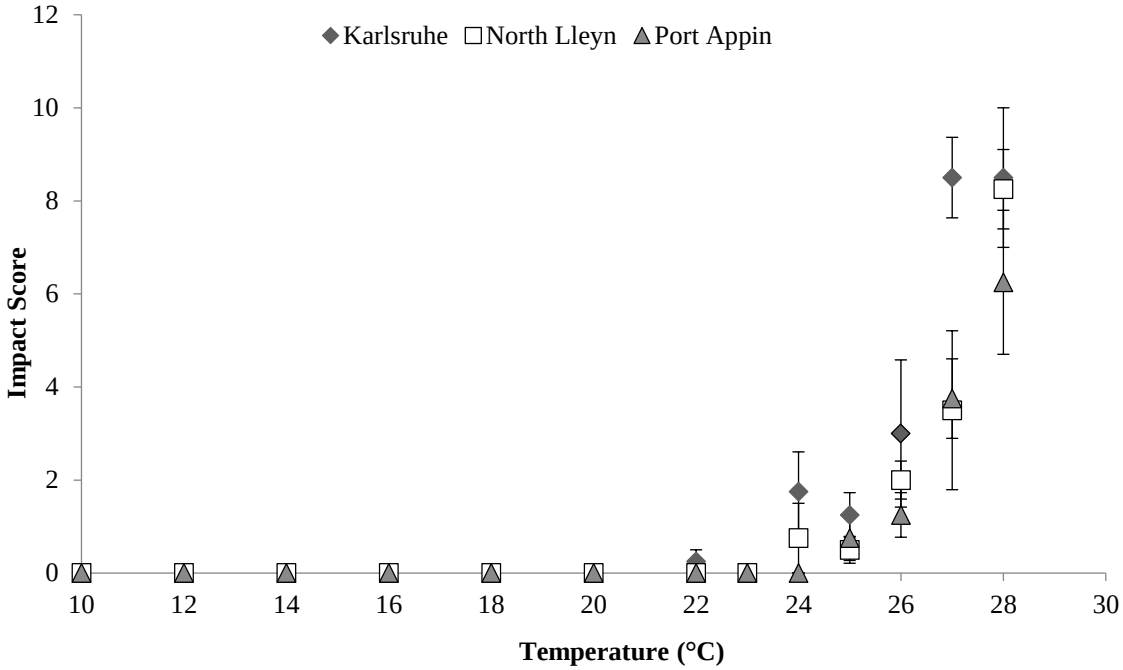


Figure 4.18. Comparison of thermal limits of *M. modiolus* from Karlsruhe, Port Appin and North Lleyn populations. Mean impact scores ± 1SE shown.

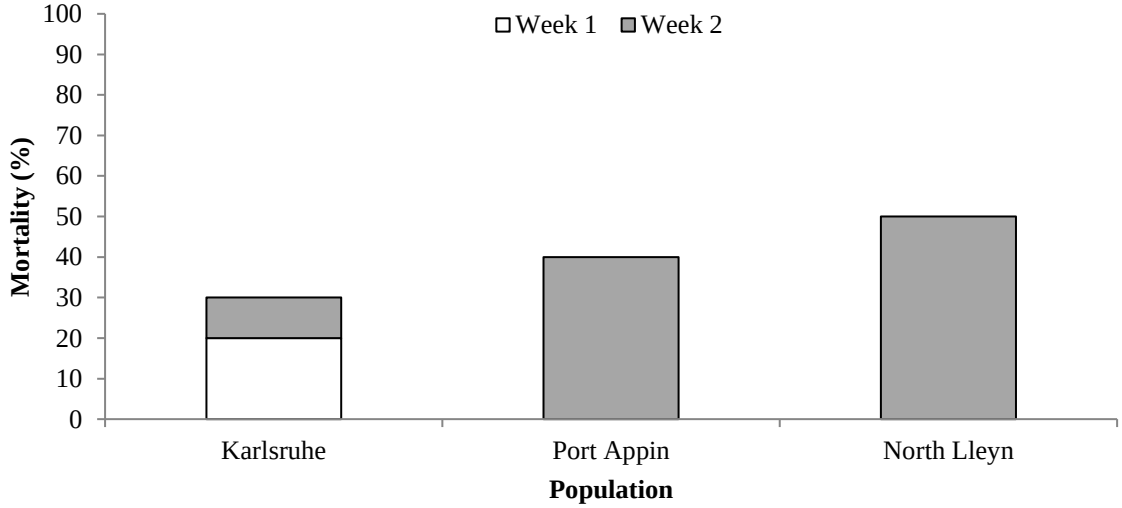


Figure 4.19. Post-experimental mortality (cumulative % after 1- and 2-week recovery period) of *M. modiolus* from Karlsruhe, Port Appin and North Lleyn populations.

Experiment 2. Effect of hypoxia to thermal limits in a *M. modiolus* reef population at the southern limit of the species' range

Under ambient temperature (10°C), both normoxic and hypoxic control groups showed no decreased ability to respond (impact scores = 0). LT50 did not vary between hypoxic and normoxic groups under warming conditions. Repeated measures GLM analyses showed no difference between impacts scores of hypoxic and normoxic groups over time, under warming conditions. However, under warming conditions, animals under hypoxic conditions had a significantly higher ($F_{(1,9)}$=6.00, p=0.040) impact score at 24°C than those under normoxic conditions, the latter not showing any decreased response until 26°C (Figure 4.20). Following one week recovery at 11°C and fully oxygenated waters, animals exposed to coinciding warming and hypoxic conditions showed increased mortality (40%) as compared to those exposed to warming alone (6.7%). Mortality for both groups were high after two weeks, but animals exposed to coinciding warming and hypoxic conditions still showed increased mortality (67%) as compared to those exposed to warming alone (60%). There were no mortalities for the control groups (Figure 4.21).

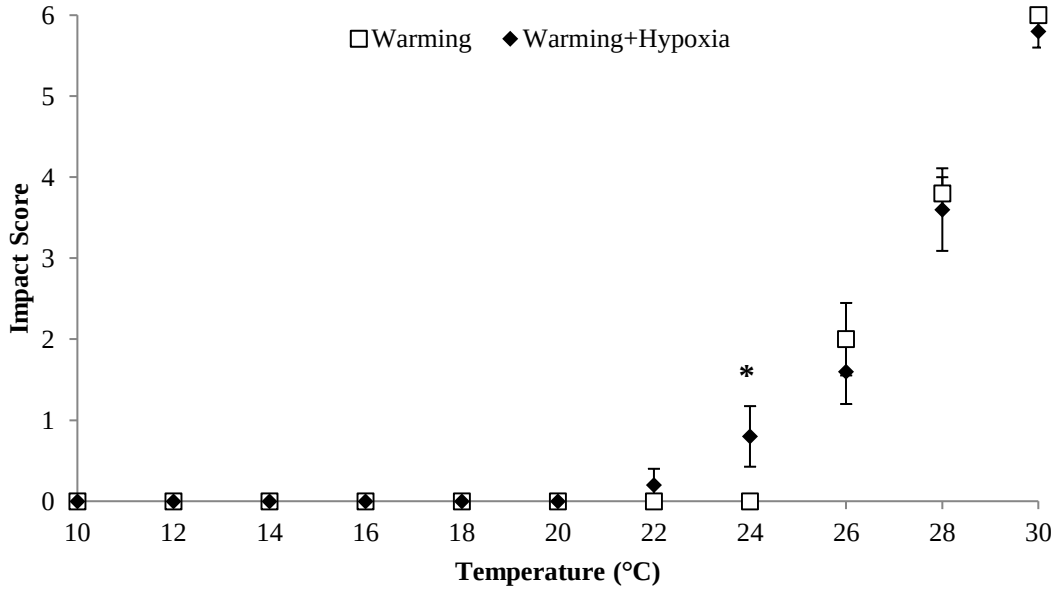


Figure 4.20. Thermal limits of *M. modiolus* (North Lleyn population) under normoxic (warming) and hypoxic (warming+hypoxic) conditions. Mean impact scores ± 1SE shown. * indicates a significant difference between normoxic and hypoxic groups.

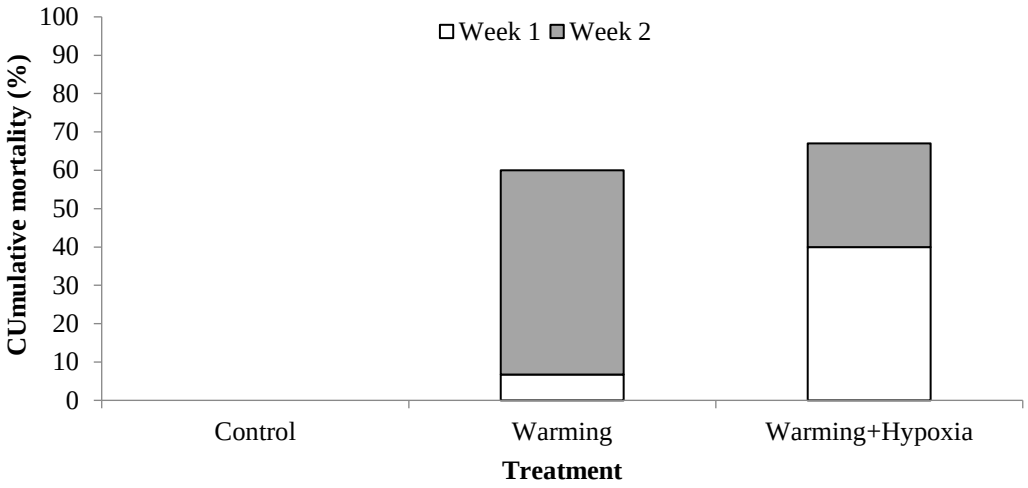


Figure 4.21. Post-experimental mortality (cumulative % after 1- and 2-week recovery period) of *M. modiolus* (North Lleyn population) for control (animals held at ambient temperature), warming and warming+hypoxia treatment animals.

Experiment 3. Oxidative stress response in a *M. modiolus* reef population under acute warming conditions

DNA damage

Results confirmed no significant difference in viability of cells between treatments (Figure 4.22). Likewise, there was no significant effect of acute warming to DNA damage in haemolymph or gill. However, there was a significantly higher ($F_{(1,4)}$=12.330, p=0.017) amount of DNA damage in gills cells in the warming group under 10°C (i.e. at the beginning of the trial) (Figure 4.23). Experimental values (means±1SE) are provided in Appendix B, Table B2.

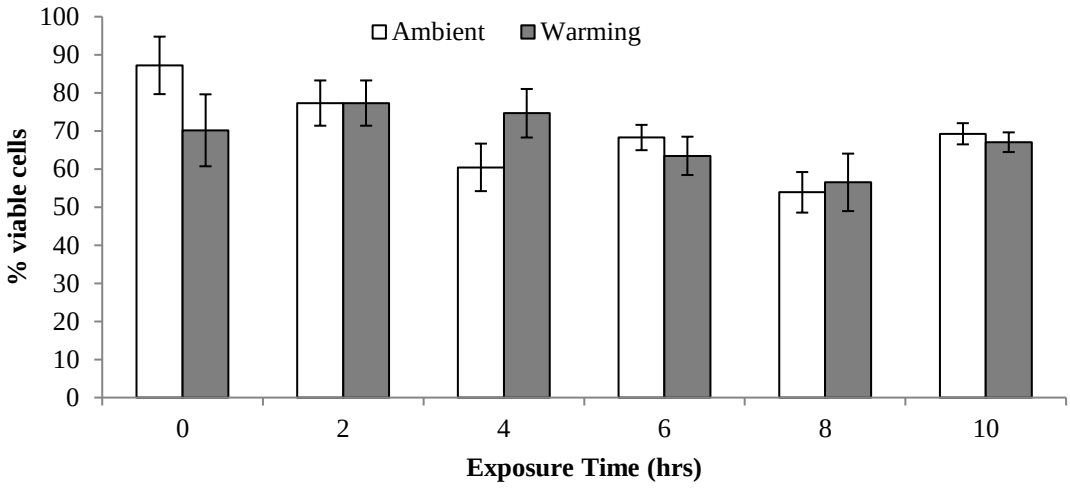


Figure 4.22. Cell viability of haemolymph cells of *M. modiolus* (Karlsruhe population) following acute temperature stress (10°C in 10 hours).

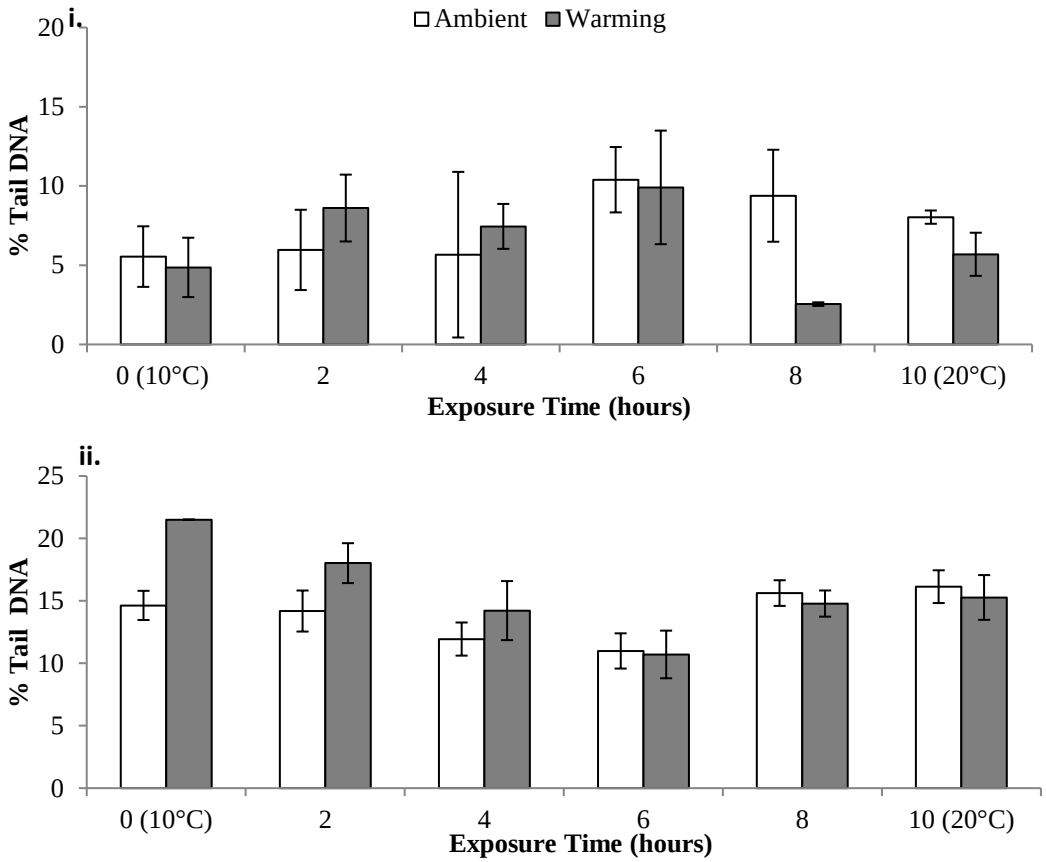


Figure 4.23. DNA damage (% Tail DNA) in (i) haemolymph and (ii) gill tissues of Karlsruhe *M. modiolus* following acute temperature stress (10°C in 10 hours).

Lipid peroxidation

No significant differences in lipid peroxidation were detected between ambient and warming groups (Figure 4.24). Experimental values (means±1SE) are provided in Appendix B, Table B2.

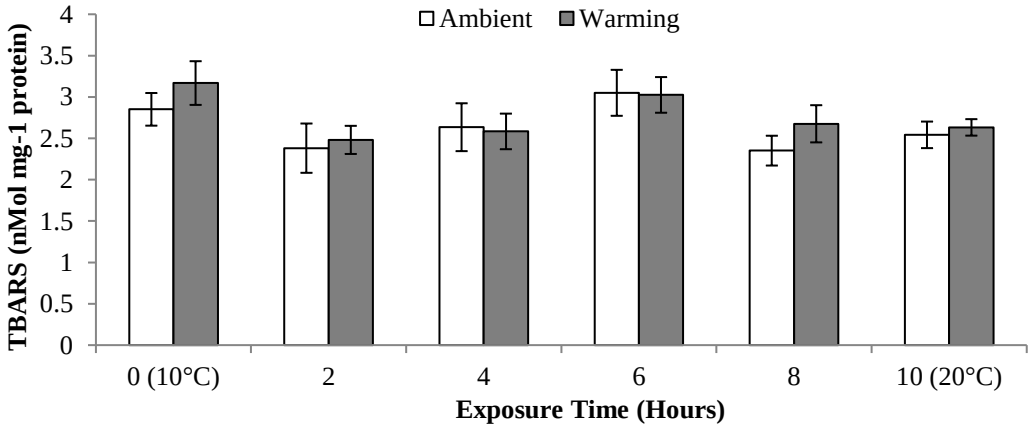


Figure 4.24. Lipid peroxidation (expressed as TBARS) in gill tissues of Karlsruhe *M. modiolus* following acute temperature stress (10°C in 10 hours).

Experiment 4. Comparison of oxidative stress response in *M. modiolus* reef populations under short-term exposure to site-specific warming conditions

DNA damage

There was no significant effect of warming to DNA damage in either haemolymph or gill cells in Karlsruhe or Port Appin populations. In the North Lleyn population, there was no significant effect of warming to DNA damage in haemolymph cells but a significant effect to gill cells $F_{(2,12)}$=8.491, p=0.005. Post hoc tests showed significantly higher levels of damage in gill cells under warming conditions as compared to the control group (p=0.006) and baseline group (p=0.018). Comparisons of effects between populations showed significantly increased levels of DNA damage in gill cells of the North Lleyn populations ($F_{(2,12)}$=8.491, p=0.004) as compared to Karlsruhe (p=0.004) and Port Appin (p=0.019) populations (Figure 4.25).

Lipid peroxidation

There was no significant effect of warming to lipid peroxidation in North Lleyn or Port Appin populations. Karlsruhe showed a significant decrease in lipid peroxidation under warming conditions ($F_{(1,12)}$=4.499, p=0.049) from control values (p=0.043) (Figure 4.26).

SOD

No significant differences were found between baseline, control or warming groups for any population, nor between populations across treatments (Figure 4.26).

Experimental values (means±1SE) for all oxidative stress biomarkers are provided in Appendix B, Table B3.

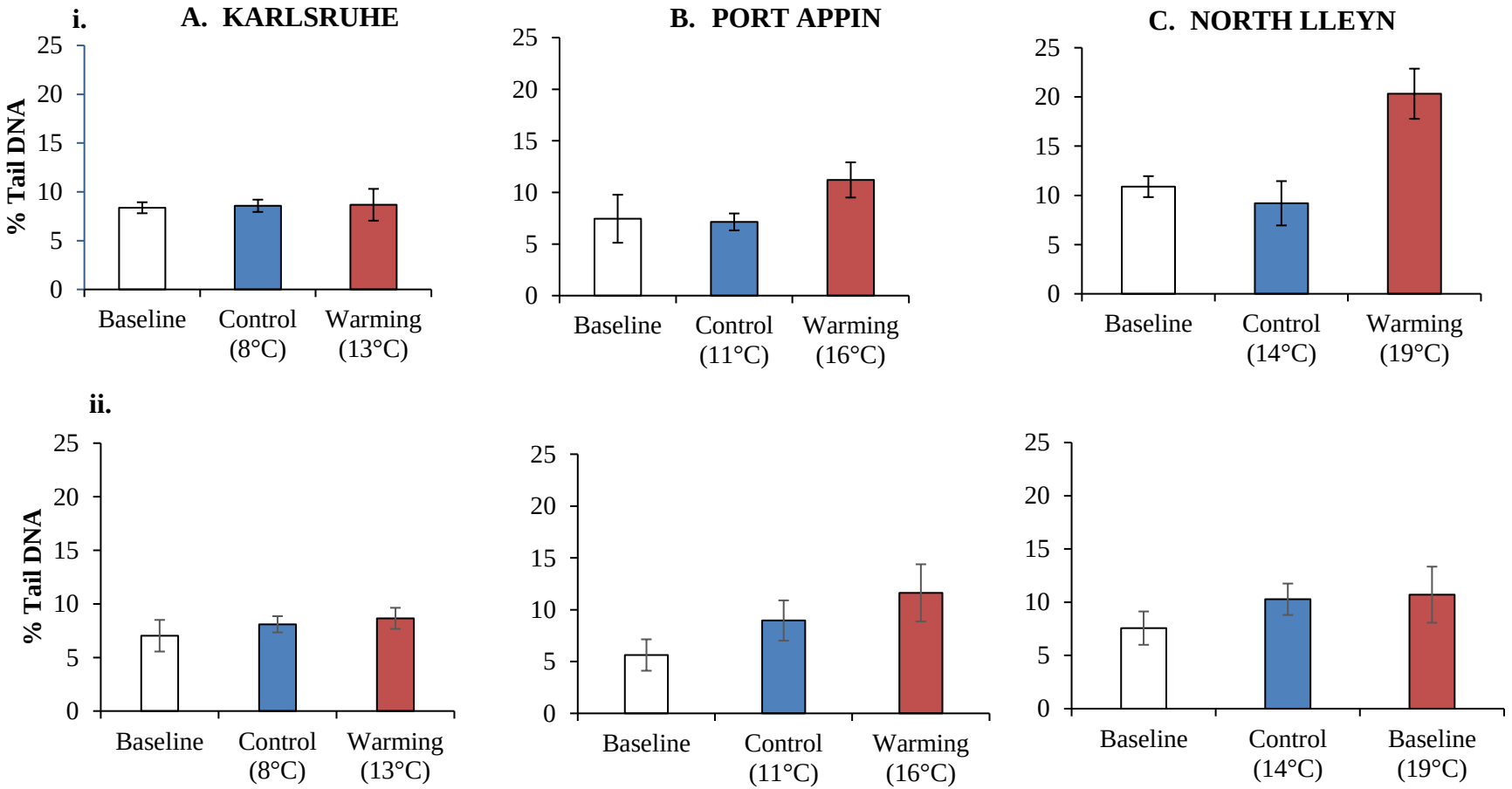


Figure 4.25. DNA strand break (expressed as % tail DNA) in (i) gill and (ii) haemolymph cells of *M. modiolus* populations (a. Karlsruhe, b. Port Appin, c. North Lleyn) following exposure to ambient (collection temperature) and warming (ambient + 5°C) conditions. Means ± 1SE shown for n=5.

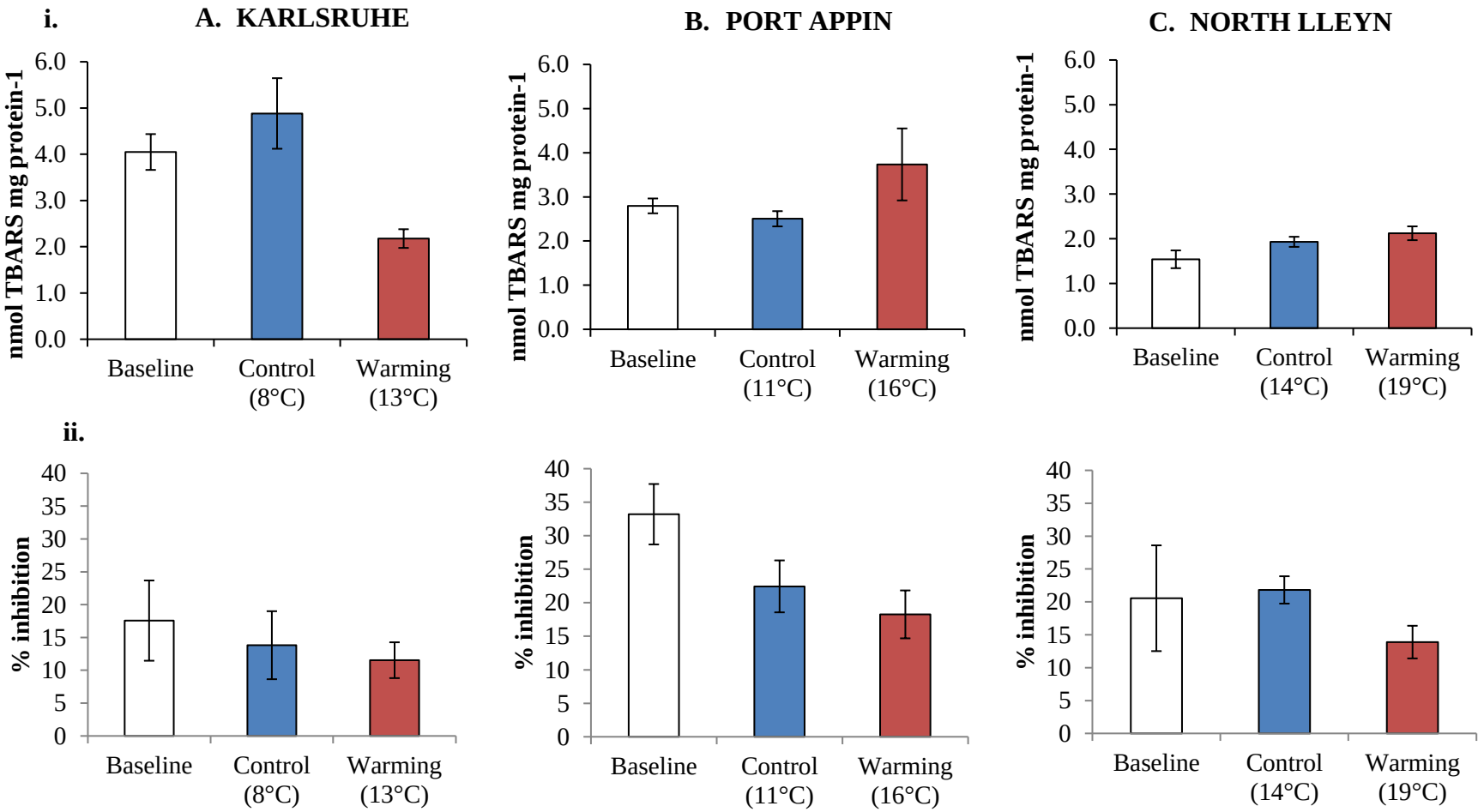


Figure 4.26. (i) Lipid peroxidation (expressed as concentration of TBARS per mg protein) and (ii) Superoxide Dismutase (SOD) (expressed as % inhibition) in *M. modiolus* populations (a. Karlsruhe, b. Port Appin, c. North Lleyn) following exposure to ambient (collection temperature) and warming (ambient + 5°C) conditions. Means ± 1SE shown for n=5.

Experiment 5. Oxidative stress response in a *M. modiolus* reef population under short-term exposure to hypoxia

There were no significant differences in TBARS or SOD across all groups or between hypoxia and normoxic groups within post-recovery or post-exposure groups. Significant differences in SOD ($F_{(1,18)}$=15.682, p=0.001) and protein carbonyl levels ($F_{(1,18}$=20.723, p<0.001) were detected between post-exposure and post recovery groups. Additionally, the post-exposure group exposed to normal oxygen conditions had significantly higher ($F_{(4,20)}$=10.463, p<0.001) concentrations of protein carbonyl than any other group (Baseline: p=0.02, post-exposure hypoxia: p=0.022; post-recovery both <0.001) (Figure 4.27). Experimental values (means±1SE) for all oxidative stress biomarkers are provided in Appendix B, Table B4.

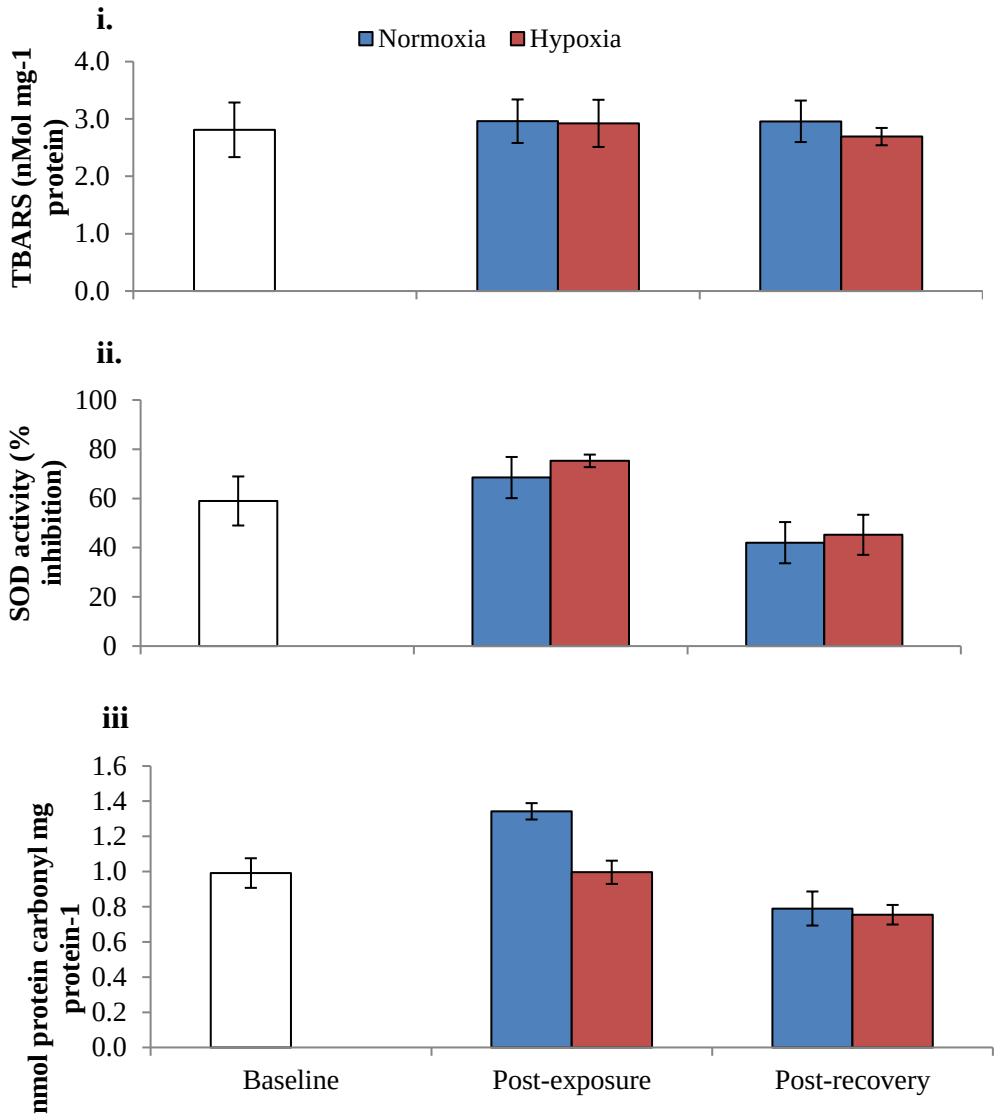


Figure 4.27. Oxidative stress response in Ramsey Bay (Isle of Man) *M. modiolus* reef population following one week exposure to hypoxic conditions (post-exposure) and following 6 hours of recovery under normal oxygen conditions (post-recovery): (i) lipid peroxidation; (ii) SOD activity; (iii) protein carbonylation. Means±1SE presented for n=5.

Experiment 6. Comparison of oxidative stress response in northern and southern *M. modiolus* reef populations under medium-term exposure to warming and hypoxia conditions

Results are presented for DNA damage (in gill and haemolymph cells), lipid peroxidation, catalase, SOD and protein carbonylation biomarkers for Karlsruhe and North Lleyn populations in parallel to provide illustration of potential population-based differences in response to warming/hypoxia stressors. Experimental values (means±1SE) for all oxidative stress biomarkers are provided in Appendix B, Table B5.

DNA damage

Haemolymph cells

Haemolymph DNA damage data for Karlsruhe *M. modiolus* was log10 transformed to meet parametric assumptions of homogeneity of variances. There was a significant effect of temperature ($F_{(2,27)}$=8.068, p=0.002) to DNA damage in haemolymph cells of Karlsruhe *M. modiolus* but no effect of oxygen content ($F_{(1,28)}$=2.329, p=0.141) nor interaction between the two factors ($F_{(2,27)}$=0.180, p=0.837). Post hoc showed significant increased DNA damage under 18°C as compared to 10°C (p=0.004) or 14°C (p=0.007) (Figure 4.28).

Raw data for North Lleyn haemolymph samples were lost so results here are as reported by Smedley (2015) who completed the work for his MSc thesis. A significant effect ($p < 0.05$) of temperature to DNA damage in haemolymph cells was detected but there was no effect of oxygen content (p=0.288) nor significant interaction of the two factors (p=0.119). A one-way ANOVA detected a significant difference between treatments and the baseline ($P < 0.01$). Post hoc (Tukey's HSD) showed that treatments 14N, 18N and 18H had significantly greater damage than the baseline sample ($p<0.05$). A significant difference ($P < 0.05$) between 10N with 18N/ 18H, 10H with 18N/ 18H, 14N with 18H and 14H with 18N/ 18H was also detected (Smedley, 2015) (Figure 4.28).

Gill cells

There was no significant effect of temperature ($F_{(2,27)}$=2.526l, p=0.118) or oxygen content ($F_{(1,28)}$=3.448, p=0.086) to DNA damage in gill cells of Karlsruhe *M. modiolus* nor interaction between the two factors ($F_{(2,27)}$=0.455, p=0.644) (Figure 4.28). Comet assay gill data for North Lleyn *M. modiolus* failed parametric requirements for homogeneity of variances even after data transformation. 2-way ANOVA indicated a significant effect

of temperature ($F_{()}$=20.517, p<0.001) and oxygen content ($F_{(1,28)}$=48.238, p<0.001) to DNA damage in gill tissue but no significant interaction between the two factors ($F_{2,27)}$=2.496, p=0.105). Post hoc tests indicated that animals held at 18°C had significantly higher DNA damage in gill cells than those held at 14°C (p<0.001) or 10°C (p<0.001) (Figure 4.28).

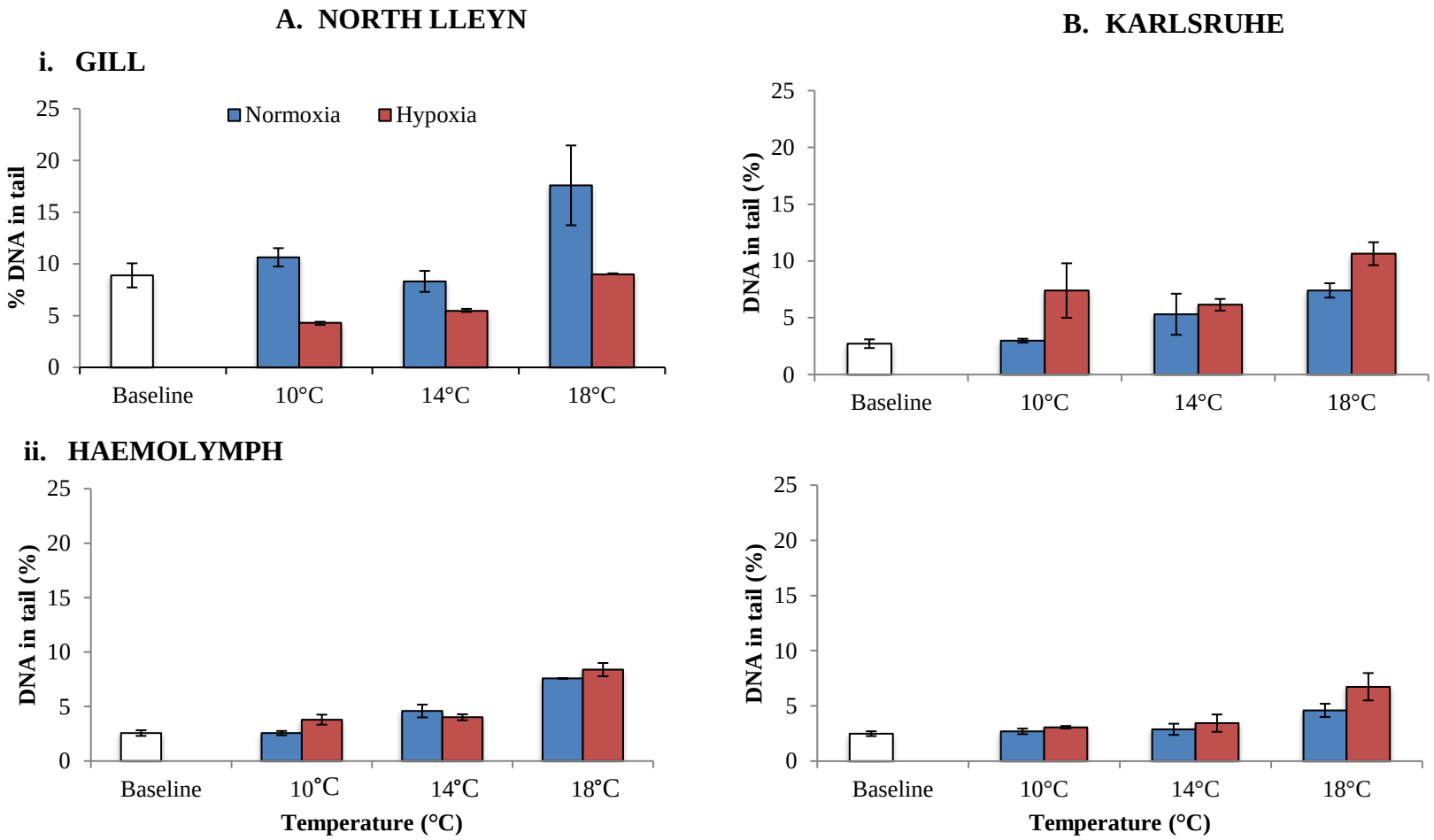


Figure 4.28. DNA strand break (expressed as % tail DNA) in (i) gill and (ii) haemolymph cells of (A) North Lleyn and (B) Karlsruhe *M. modiolus* populations following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia/hypoxia. Means±1SE presented for n=5.

Lipid Peroxidation

TBARS data were log transformed for both North Lleyn and Karlsruhe populations to meet parametric assumptions of homogeneity of variance. There was a significant negative effect of oxygen content ($F_{(1,28)}$=18.048, p<0.001) to production of TBARS in Karlsruhe *M. modiolus* but no effect of temperature ($F_{(2,27)}$=1.201, p=0.321) nor interaction between the two factors ($F_{(2,27)}$=0.610, p=0.553). There was a significant positive effect of temperature ($F_{(2,27)}$=5.687, p=0.010) to production of TBARS in North Lleyn *M. modiolus* but no effect of oxygen content ($F_{(1,28)}$=0.509, p=0.483) nor interaction between the two factors ($F_{(2,27)}$=1.016, p=0.377). *M. modiolus* held at 18°C had significantly higher TBARS than those held at 10°C (p=0.007) (Figure 4.29).

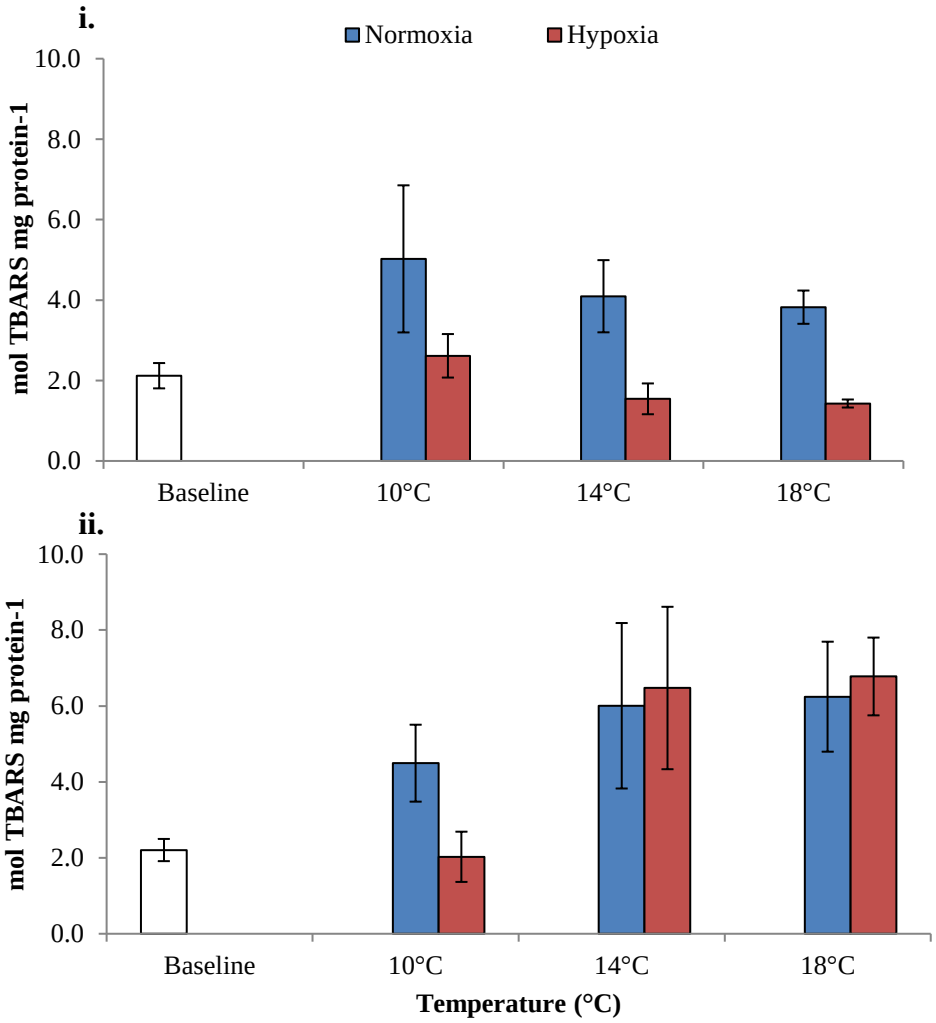


Figure 4.29. Lipid peroxidation (as production of TBARS) in (i) Karlsruhe and (ii) North Lleyn *M. modiolus* populations following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia/hypoxia. Means±1SE presented for n=5.

SOD

There was a significant negative effect of temperature ($F_{(2,27)}$=3.709, p=0.039) to SOD production in Karlsruhe *M. modiolus* but no effect of oxygen content ($F_{(1,28)}$=3.930, p=0.059) nor interaction between the two factors ($F_{(2,27)}$=2.445, p=0.108). Post hoc testing showed that animals held at 18°C had significantly lower SOD levels than those at 14°C (p=0.031). There was no effect of temperature ($F_{(2,27)}$=0.039, p=0.962) or oxygen content ($F_{(1,28)}$=0.097, p=0.758) to SOD production in North Lleyn *M. modiolus* nor interaction between the two factors ($F_{(2,27)}$=0.666, p=0.523) (Figure 4.30).

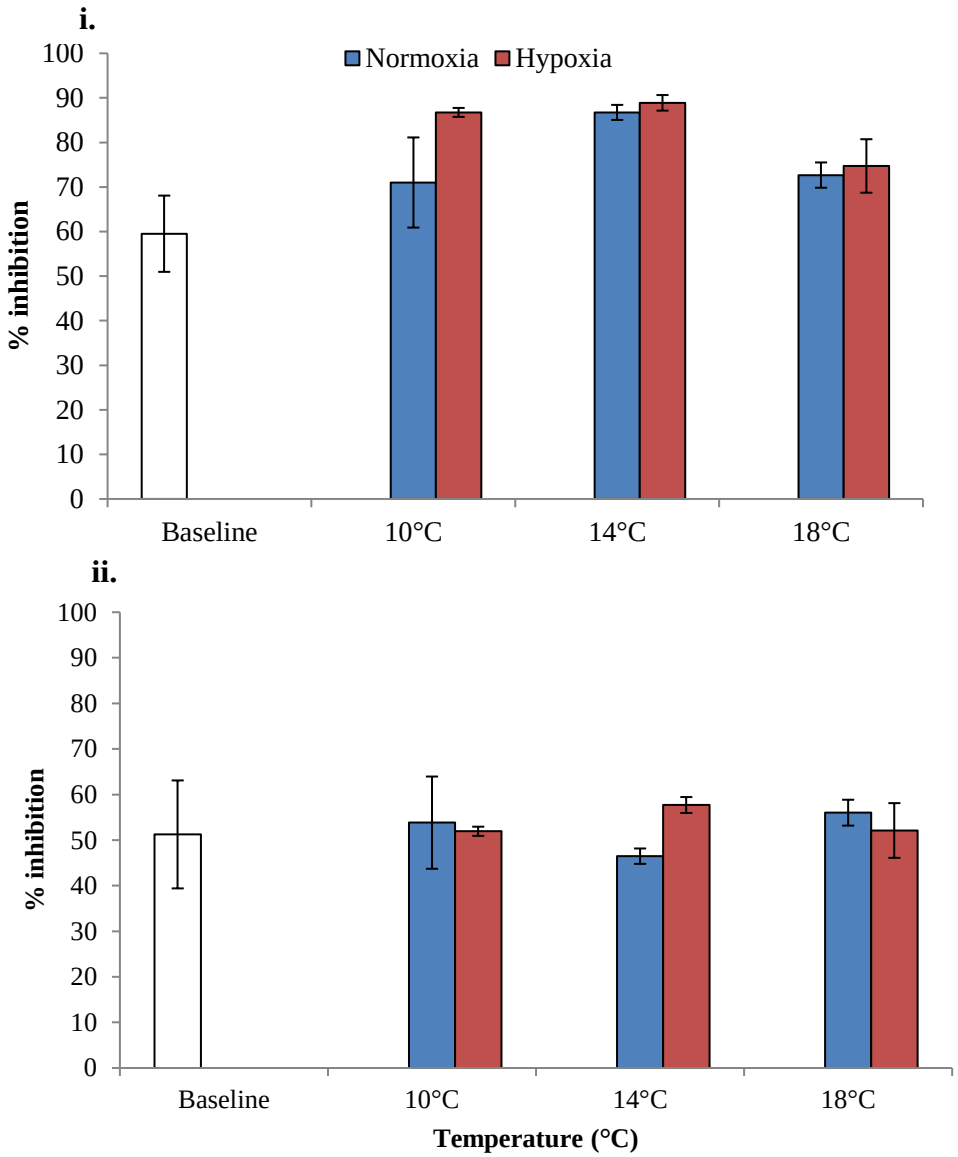


Figure 4.30. SOD production (% inhibition of oxidation) in (i) Karlsruhe and (ii) North Lleyn *M. modiolus* populations following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia/hypoxia. Means±1SE presented for n=5.

Protein Carbonylation

There was a significant effect of hypoxia to protein carbonylation in Karlsruhe *M. modiolus* ($F_{(1,28)}$=15.995, p=0.001); but no effect of temperature (F=3.078, p=0.065) nor interaction ($F_{(2,27)}$=2.161, p=0.138). However, data failed to meet parametric assumptions of homogeneity of variance even after transformation. There was no significant effect of temperature ($F_{(2,27)}$=3.190, p=0.062) or hypoxia ($F_{(1,28)}$=0.000, p=0.985) to protein carbonylation in North Lleyn *M. modiolus* nor a significant interaction ($F_{(2,27)}$=0.210, p=0.812) between the two factors (Figure 4.31).

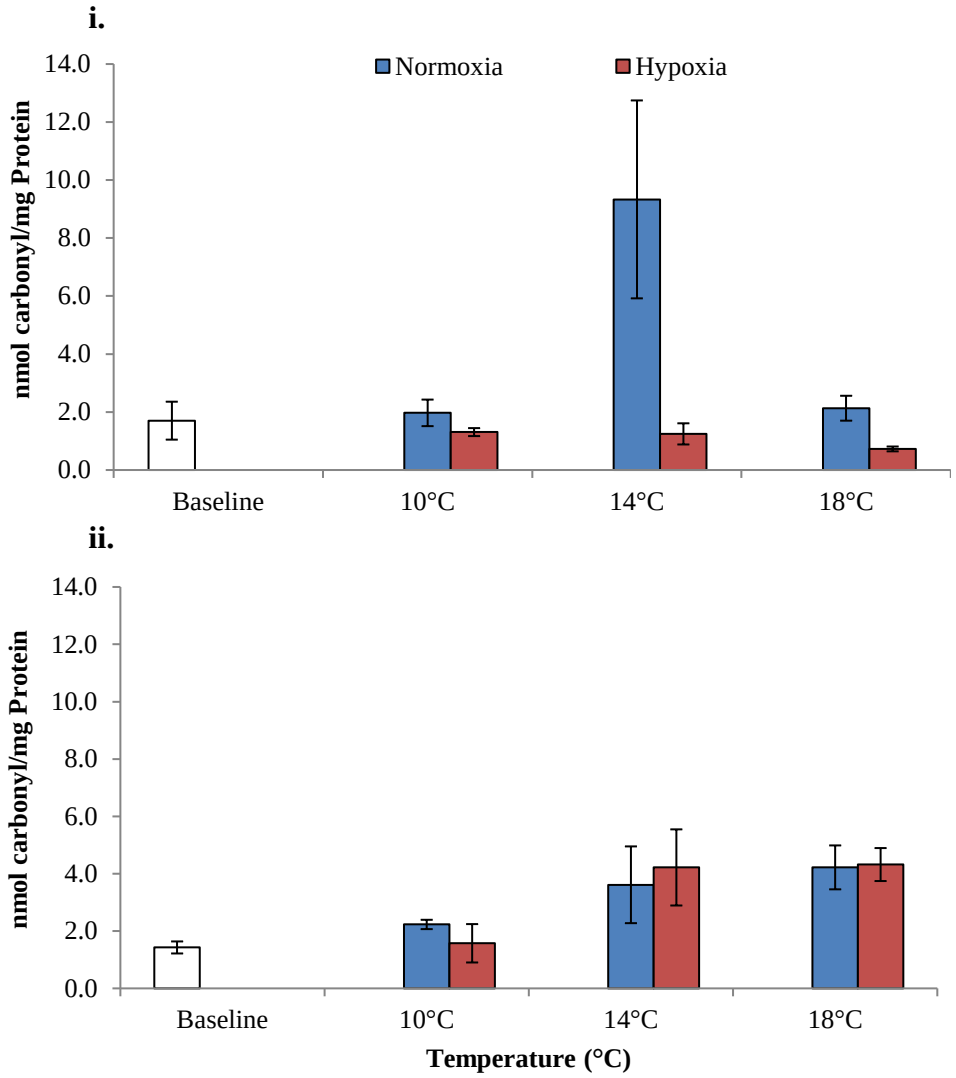


Figure 4.31. Protein carbonylation in (i) Karlsruhe and (ii) North Lleyn *M. modiolus* populations following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia/hypoxia. Means±1SE presented for n=5.

Catalase

Catalase data for North Lleyn and Karlsruhe *M. modiolus* was log10 transformed to meet parametric assumptions of homogeneity of variance. There was a significant (F=27.979, p=<0.001) positive effect of oxygen content to catalase production in Karlsruhe *M. modiolus* but no effect of temperature (F=1.590, p=0.225) nor interaction between the two factors (F=0.620, p=0.546). There was a significant positive effect of temperature to catalase production in Wales *M. modiolus* (F=5.532, p=0.011) but no effect of hypoxia (F=0.139, p=0.712) nor interaction between the factors (F=1.069, p=0.359). Post hoc indicated that animals held at 18°C had significantly higher production of catalase than those at 10°C (p=0.008) (Figure 4.32).

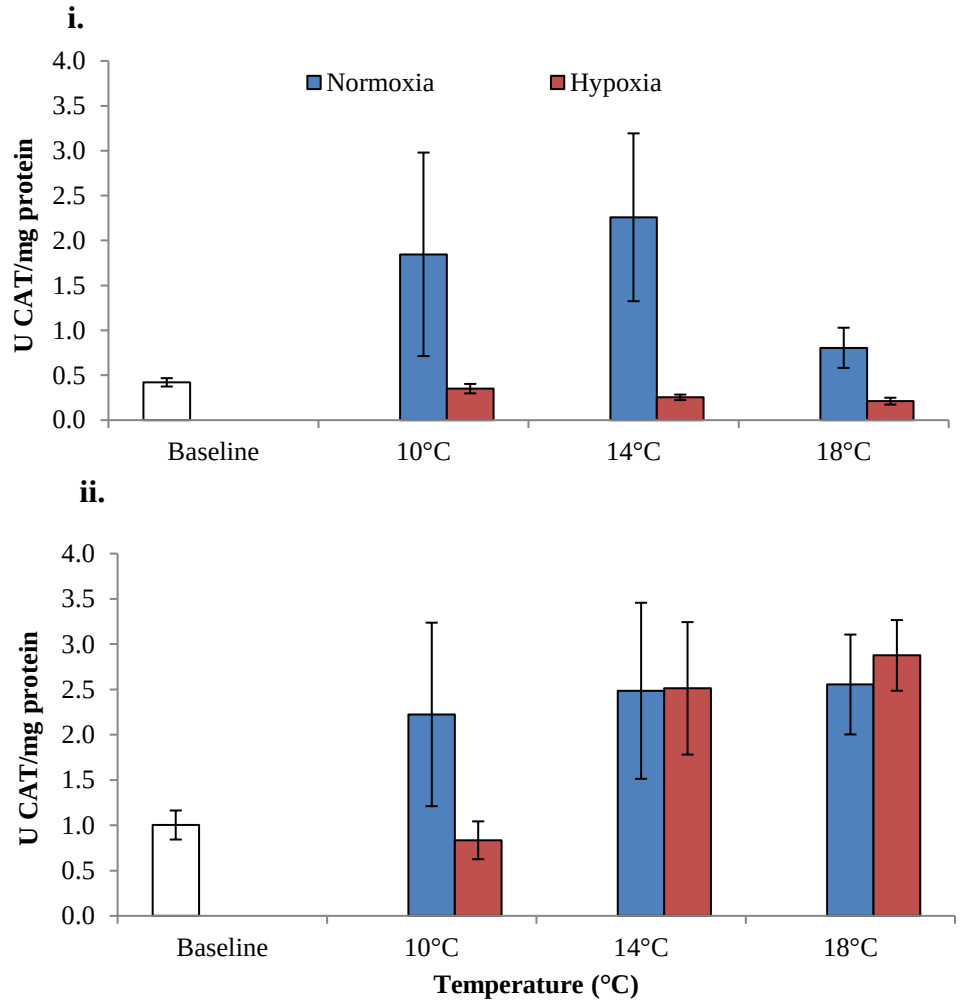


Figure 4.32. Catalase production in (i) Karlsruhe and (ii) North Lleyn *M. modiolus* populations following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia/hypoxia. Means±1SE presented for n=5.

4.6 Discussion

During this investigation, initial findings were generated regarding the thermal limits and oxidative stress response to climate change stressors in *M. modiolus* at species- and population-level. Impacts varied according to exposure type, duration and intensity, and overall warming appears to be a more serious threat to *M. modiolus* than hypoxia with minimal interaction between the two factors. However, data did suggest that specific populations may be more sensitive to one type of stressor than another. Further, results provide preliminary indication that populations may have varying acclimatisation potential and therefore be at varying threat of climate change.

Thermal limits

Results indicate a LT50 for *M. modiolus* of approximately 27-28°C. Thermal tolerance limits for *M. modiolus* have previously been approximated between 16-20°C (Halanych *et al*., 2013). Further, Read & Cumming (1963) reported that *M. modiolus* is restricted in its natural distribution by upper limits of 23°C. In comparison, thermal limit values determined in the current study seem high. While repeated experimentation would be necessary for confirmation of true thermal limits, it is suggested that the substantial post-exposure mortality observed across all populations could mean that thermal limits recorded here were into the lethal range for the species (i.e. beyond "limits"). Given that *M. modiolus'* internal temperatures may take some time to reach equilibrium with external environments (Pianka, 1970; Helmuth, 1998), impacts recorded at a given temperature could be a delayed response to an earlier temperature, and thus provide an exaggerated measure of the true thermal limits for the species.

While absolute thermal limits for *M. modiolus* remain uncertain, comparison of limits between populations indicated slight shifts in LT50 between populations. Findings may therefore provide insight into relative population-based sensitivities to warming. Further, measurements may indicate the relative degree of fitness of different populations over their geographical distribution, and help to pinpoint priority populations with regards monitoring under climate change (Southward *et al*., 1995). Temperature of first impact (i.e. decreased response) varied across populations, with the northernmost population (Karlsruhe) appearing to have the lowest tolerance to increased temperature. Karlsruhe showed first decreases in response at a lower temperature, reached LT50 at a lower temperature, and had the highest post-exposure mortality after one week. However, all

populations had considerable (30-50%) mortality after two weeks indicating a substantial negative effect of acute warming stress to the species as a whole. Moreover, the most southern population (North Lleyn) had the greatest cumulative mortality (50%) and was induced to spawn at a lower temperature and across a higher number of tanks than Port Appin or Karlsruhe populations. Subsequently, despite a higher LT50 than the most northern population, North Lleyn *M. modiolus* arguably demonstrated the strongest negative response to warming.

While species may have physiological mechanisms (discussed below) to cope with oxidative stress damages brought on by thermal stress conditions, the recovery period following such events can be further damaging as the re-oxygenation of formerly oxygen-deprived tissues leads to elevated ROS production (Heise *et al*., 2006). This has been observed in intertidal bivalve species where low tissue oxygen levels brought on by shell closure during immersion are followed by a rapid increase in oxygen during emersion, and a parallel burst of ROS production (Sheehan & McDonagh, 2008). However, intertidal species deal with such fluctuations on a daily basis and thus likely possess a sufficient degree of plasticity to cope with ROS fluxes. In contrast, *M. modiolus* has limited regular exposure to such oscillations and thus ROS production associated with the recovery period could have consequences for survival. Consequently, thermal stress events may have severe carry-over effects, and could account for the high level of post-exposure mortality observed across all populations.

Upper thermal limits tend to decrease with an increase in latitude between populations of the same species (Spicer & Gaston, 1999; Logan *et al*., 2012), and consequently it is not surprising that the Karlsruhe population, as the northernmost group, demonstrated increased sensitivity to thermal stress. However, under the same logic, one would expect the population at the southern limit of the range (i.e. North Lleyn) to have the highest thermal limits. However, the mid-latitude population (Port Appin) showed consistently lower mean impact scores than the North Lleyn population, suggesting a higher upper thermal limit. Previous discussion of historical conditions at west coast sites (refer to Chapter 2) showed that enclosed loch sites have decreased water exchange and are exposed to warmer temperatures across the year. Consequently, the Port Appin population may experience historical exposure to increased temperatures and therefore be better acclimatised to cope with warming conditions. Likewise, the Port Appin

population may regularly face hypoxic events, and thus may have physiological strategies or plasticity to contend with low oxygen levels that are characteristic of thermal stress. Results highlight the importance of considering the shaping influence of site specific abiotic histories to respective resiliencies (refer to Chapter 2).

The high mortality and high occurrence of induced spawning observed in the North Lleyn population suggests that the population may have a high sensitivity to warming. Thermally-induced spawning is a well-documented stress response in bivalves and frequently employed in aquaculture operations to acquire larvae from brood stock (Mies & Sumida, 2012). This is somewhat alarming as the North Lleyn is situated at the southern limit of the range, so may at present already face thermal maxima at certain times of year. In addition to causing physiological stress in the population, warming-induced spawning could also lead to earlier/later annual spawning events (i.e. shifting breeding windows) and a potential mismatch between larvae and food supply (Durant *et al*., 2007), highlighting potential ecological repercussions. Additionally, warming could cause reduced gamete quality and viability (Boni *et al.*, 2016) and therefore reduced reproductive success and larval quality. Further, given that hydrodynamic processes are not static across the year (e.g. summer stratification), changes in dispersal timing or larval quality could potentially impact genetic connectivity (refer to Chapter 3).

Thermal limits tests also showed a negative influence of environmental hypoxia to thermal tolerance limits with thermal limits in the North Lleyn *M. modiolus* population lower under hypoxic conditions. Additionally, post-exposure mortality was higher under co-stressor conditions. As upper thermal limits are closely associated with warming-induced oxygen demands, results here support the theory that thermal tolerance limits are set by limited oxygen supply (Pörtner, 2002). Accordingly, in a low oxygen environment, one would expect oxygen supply to tissues to further decrease with parallel decreases to thermal limits. Accordingly, it is proposed that parallel occurrence of hypoxia under warming conditions will put *M. modiolus* at further risk via reducing thermal limits.

Oxidative Stress Response

Acute warming showed no impact to oxidative stress in *M. modiolus* suggesting that the species may have physiological mechanisms for coping with short-term thermal stress. Bivalves have demonstrated a variety of adaptions to cope with short-term oxidative

stress including depression of activity (e.g. reduced feeding activity), reduced metabolic rates, and a shift to anaerobic metabolism (David *et al*., 2005; Vaquer-Sunyer & Duarte, 2008). While it remains to be discovered whether *M. modiolus* can use such mechanisms to specifically counteract oxidative stress, the species has been shown to alter metabolic and respiration rates, adjust feeding rates and modify the activity of metabolic rate-limiting enzymes in response to seasonal differences in temperature (Navarro & Thompson, 1996; Lesser & Kruse, 2004). Likewise, a southern congener (*Modiolus barbatus*) has demonstrated metabolic depression and a shift from aerobic to anaerobic metabolism under thermal stress conditions (Anestis *et al*., 2008).

Differences in growth rates parallel differences in oxidative stress across numerous animal taxa with slowed growth rates associated with increased resistance to oxidative stress (Costantini, 2010). Conversely, rapid growth has been suggested to cause increased oxidative damage due to higher metabolic rates which increase production of ROS, and further, because rapid growth diverts resources away from maintenance and repair of molecular damage (Lee *et al*., 2012). Additionally, low growth rates in ectotherms are associated with elevated rates of protein turnover. Increases in protein synthesis are linked with an increased ability to survive environmental stress events as higher rates allow for protein remodelling, flexibility in enzyme production and the repair of damaged proteins (Rastrick & Whiteley, 2013). Thus, as a species characterised as slow-growing (Navarro & Thompson, 1996), *M. modiolus'* growth rates may afford it some advantage towards coping under short-term stress. However, increased protein production also cause considerable increases in energy expenditure (Rastrick & Whiteley, 2013). Consequently, under extended exposure to stressor conditions, it is likely that curtailing of impacts would be unsustainable, particularly as impacts to energy budgets would probably have knock-on effects to maintenance of physiological homeostasis (refer to Chapter 5). *M. modiolus* is also characterised as relatively long-lived, with many individuals living in excess of 30 years (Fariñas-Franco *et al.,* 2014). Longevity has also been associated with increased resilience to oxidative stress due to the relationship between growth rates and aging (i.e. faster growth rates = decreased longevity) (Lee *et al*., 2012).

In contrast to *M. modiolus*' reduced response under acute thermal stress, extended thermal exposures led to increases in oxidative stress including measurable increases in DNA

damage and lipid peroxidation. This may suggest that *M. modiolus* is unable to sustain any of the proposed fore-mentioned physiological adaptations that might allow it to tolerate short-term stress conditions, and that extended exposure will lead to increased oxidative damage. While bivalves may use metabolic depression to conserve energy and minimise disruption of cellular redox under stressor conditions, such response is typically only sustainable in the short-term, and longer-term exposures have been shown to lead to increases in metabolic rates of bivalves (Thomsen & Melzner, 2010). ROS are produced as side-products of oxygen metabolism, and thus, increases in metabolism have direct consequences for oxidative stress levels (Lushchak, 2011). Further, increased metabolic rates increase cellular energy demands, at the detriment of defence and repair mechanisms (Lesser, 2006).

However, while warming led to impacts to DNA and lipids, no damages to proteins (i.e. protein carbonylation) were detected. Mattoo *et al*. (2013) also found no effect of moderate warming (+5°C) to protein carbonylation in two bivalve species (*Crassostrea virginica* and *Mercenaria mercenaria*), despite increases in standard metabolic rates indicating increased stress. Such decreases could be a result of declines in rates of protein synthesis that occur with warming (Pörtner, 2002). Therefore, a lack of effect to protein carbonylation could either imply that the species has not been impacted or, in contrast, indicate a reduced ability to carry out fundamental cellular growth processes. Consequently, protein carbonylation may not be an appropriate biomarker for measuring the effects of thermal stress.

Consideration of the sole effects of hypoxia to oxidative stress in *M. modiolus* showed minimal effect of low oxygen conditions. However, as response to hypoxia depends on persistence and timing of hypoxia (Levin *et al*., 2009), the relatively short lengths of hypoxic exposures investigated here (i.e. one day to one week maximum duration) may account for the lack of impact. In contrast, review of median lethal times of hypoxia exposure by Vaquer-Sunyer & Duarte (2008) showed lethal exposures for molluscs as high as 32 weeks in the bivalve *Astarte borealis*. Further, comparison of marine taxa has shown that molluscs are highly tolerant to hypoxia (Vaquer-Sunyer & Duarte, 2008) with some species able to tolerate a wide range of concentrations of oxygen (i.e. are euryoxic) and therefore survive regular fluctuations between hypoxia/anoxia and normoxia. Euryoxic species can also maintain low levels of ROS during transition between oxygen

levels, and therefore avoid oxidative stress (Lesser, 2006). This has been especially noted in intertidal bivalve species that contend with daily oscillations in oxygen availability, but could potentially extend to species inhabiting sub-tidal areas where regular hypoxic events occur (e.g. sea lochs). Additionally, bivalves have the capacity to limit the activity of enzymes and proteins via reverse protein phosphorylation during low oxygen exposures (David *et al.*, 2005). While there is currently a lack of supporting literature regarding the specific ability of *M. modiolus* to apply such strategies to combat hypoxia, similar investigation into thermal impacts indicates that the species does have ability to regulate metabolism in response to temperature change (Navarro & Thompson, 1996). Consequently, the species may have some physiological flexibility to cope with low oxygen events, but further research is needed in order to understand the physiological mechanisms that might increase resilience.

There was little interaction between temperature and oxygen content, suggesting minimal synergistic effect of the two stressors to *M. modiolus*. Further, and surprisingly, there was negative effect of hypoxia to oxidative damage following several weeks' exposure to warming, with effect varying between populations. Hypoxia conditions caused decreased lipid peroxidation in the northern population (Karlsruhe) and decreased gill DNA damage in the southern population (North Lleyn). Additionally, decreases in protein carbonylation were detected under hypoxia in the Karlsruhe population, indicating that the lower oxygen environment may (in the short-term) prevent damages. The observed decreases in damages under hypoxia conditions could be a direct response to lowered oxygen conditions. Previous investigations have shown that animals may enter a metabolically depressed state during periods of low oxygen, with metabolic rates reduced to as much as 10% of normal values, and with parallel cessation of ROS formation (Philipp *et al*., 2012). Observed responses could therefore be an indication of *M. modiolus* entering aestivation (i.e. a state of hypo-metabolism or dormancy) as has been previously observed in marine organisms when a co-stressor condition occurs under thermal stress (Guderely *et al*., 2002).

Antioxidant Defences

Results suggest that *M. modiolus* populations have varied ability to counteract oxidative stress via antioxidant activity. Changes in antioxidant activity were observed to increase, decrease or remain unchanged under warming and/or hypoxia conditions, with response

dependent on antioxidant, stressor and population. For example, hypoxia caused a decline in catalase in Karlsruhe *M. modiolus* while warming lead to an increase of the antioxidant in the North Lleyn population. Likewise, while increased temperature had a significant negative effect to SOD production in Karlsruhe *M. modiolus*, there was no change in SOD activity in the North Lleyn population.

Typically, lower latitude species show improved physiological capacity to compensate for thermal stress (Whiteley & Mackenzie, 2016). For example, comparison of oxidative stress between an invasive southern (*Mytilus galloprovincialis*) and a native northern (*Mytilus californianus*) mussel species illustrated considerable variation in level of response, with the southern species demonstrating a stronger repair ability against temperature stress than its northern counterpart (Yao & Somero, 2012). Similarly, populations distributed across a latitudinal (i.e. temperature) gradient may present varying abilities to cope with thermal stress (Osovitz & Hofmann, 2007). Previous latitudinal investigations have shown differences in respiration rates, energy budgets and heat shock protein responses (Hummel *et al*., 2000; Sorte & Hofmann, 2004; Osovitz & Hofmann, 2005) between bivalve populations of the same species, suggesting varying degrees of intraspecific phenotypic plasticity.

The observed differences in oxidative stress response between North Lleyn and Karlsruhe populations could also be a result of differing historical exposures or due to varying population demographics (e.g. growth rates). Philipp *et al*. (2012), for example observed varying antioxidant defences between populations of *Arctica islandica* from sites with historically varying thermal, oxygen and salinity conditions and possessing widely varying life spans. The short-lived population accustomed to stress conditions showed limited physiological adjustment (e.g. metabolic depression) and minimal levels of oxidative stress under low oxygen conditions. Such response was hypothesised to be due to a higher physiological flexibility and stress hardening as shaped by historical environmental conditions, but at the expense of life span (Philipp *et al.*, 2012). Similarly, though to a lesser degree, historical conditions between North Lleyn and Karlsruhe *M. modiolus* differ considerably, particularly with regards temperature (refer to Chapter 2). For example, Karslruhe *M. modiolus* could potentially have a poorer ability to produce antioxidants under stress conditions due to a historical exposure to low stress (e.g. low temperature/high oxygen) conditions. The populations also vary demographically,

having contrasting growth rates, maximum sizes and levels of recruitment (Brash *et al.*, 2017). Such aspects could partially shape population-level responses. For example, the larger shell size of the Karlsruhe population could be associated with increased limits to oxygen capacity (i.e. due to reduced surface area: volume), as compared to the North Lleyn population, and thus the population may be less suited to deal with low oxygen conditions or increased oxygen demands.

While marine invertebrate species may be able to counteract thermal/hypoxic stress via increased production of antioxidants, such defence may also be compromised as thermal limits are approached. Thus, while antioxidants act as an important defence against oxidative stress, they may ultimately be unavailable as a particular stressor escalates (Heise *et al.,* 2006). As observed in the Karlsruhe *M. modiolus* population, ectotherm species have demonstrated decreased SOD activity during warming above temperature maxima (Abele *et al*., 1998; Pörtner, 2002; Heise *et al*., 2006). Such declines may be a consequence of reduced protein synthesis at higher temperatures, thereby impacting SOD expression (Pörtner, 2002). Tomanek (2012), for example, reported a disruption of protein homeostasis including upregulation of molecular chaperones and proteasome isoforms (i.e. the protein-degradation machinery of the cell) in *Mytilus* spp. following acute thermal stress. Additionally, antioxidative enzymes may have lower stability in sub-tidal invertebrates than in species typically inhabiting the intertidal zone (Regoli *et al*., 1997 Pörtner, 2002). Consequently, results may be an indication that *M. modiolus'* subtidal environment plays a role in shaping its capacity to mount a sufficient antioxidant defence.

Seasonality is also an important consideration when considering antioxidant defences in bivalves. Viarengo *et al.* (1991), for example, observed lowered levels of activity in catalase, superoxide dismutase and gluatathione peroxidise in *M. edulis* during winter months. However, prior in-situ examination of *M. modiolus* antioxidant production showed no change in SOD levels between winter and summer months, suggesting *M. modiolus* keep levels relatively constant over the year. However, the same study reported changes in metabolic enzymes and heat shock protein levels over seasons, suggesting that *M. modiolus* may uses other protective mechanisms to combat thermal stress associate with warmer months (Lesser & Kruse, 2004).

Acclimatisation Potential and Sensitivity

Results provide preliminary indication that *M. modiolus* populations may have differing acclimatisation potential to warming and/or hypoxia conditions, but an increased number of populations need to be repeatedly tested under medium to long-term exposures to draw firm conclusions. Interestingly, the two populations included in the longer-term exposure showed considerably different responses to stress conditions, particularly in terms of the type of stressor (warming vs hypoxia) inducing damages. Comparison of populations suggests that the greatest threat to *M. modiolus* under warming conditions will likely be in the southernmost population (North Lleyn) due to site temperatures approaching thermal maxima under climate change conditions. This aligns with the ecological theory that species at the edge of biogeographic ranges are more stressed and that environmental stress defines range borders (Osovitz & Hofmann, 2007). Further, the high post-experimental mortality and high incidence of induced spawning suggest that the North Lleyn population may be more sensitive to warming, with potential impacts to both survival and reproductive success. Results highlight the importance of taking a population-level approach for determination of climate change impacts and caution against sole reliance on broadcast species-level stress response, particularly where populations are spread across a variable marine environment and may have widely varying thermal (or other stressor) histories.

Overall, results emphasise the complex nature of determining *M. modiolus*' sensitivity to climate change and highlight the need for further investigation into the influence of aspects such as climate stressors' interactions, timing/severity of exposure, and pre- and post-stress conditions. Further, the current investigation demonstrates how different types of tissues and biomarkers may illustrate varying levels of impacts. Hence it is stressed that biomonitoring efforts adopt the most suitable (e.g. gill tissue; DNA damage) to detect impacts at the earliest stage possible. Results also showed a high degree of individual variation within populations and highlight a challenge of interpopulation comparisons (i.e. variation in variability). Finally, it is proposed that future research work towards a deeper understanding of the role of abiotic factors to physiological processes in *M. modiolus.* Improved knowledge would vastly help in determining any biological adaptations that might be available to the species for contending with climate change stress. Such understanding would also contribute to an improved climate envelope for

the species under climate change (Osovitz & Hofmann, 2007), which could then feed into effective management and restoration programmes for the species.

5. **Energetic Costs:** Energy budgets in *M. modiolus* under climate change conditions

5.1 Abstract

The physiological mechanisms that enable extension of tolerance limits under climate change can be energetically demanding and may therefore come at the expense of other critical functions including growth and reproduction. Stress conditions create metabolic energy demands that may exceed energy supplied from food and/or energy stores of somatic tissues, and may also overwhelm the capacity of physiological systems and cellular mechanisms involved in maintenance of routine metabolism. Consequently, analyses of energy homeostasis may be used to assess the limits of environmental stress tolerance and hence may aid in determining the vulnerability of *M. modiolus* to future climate change conditions. In this chapter, the consequences of climate change (warming and/or hypoxia) to energy budgets are investigated in *M. modiolus* including examination of i) the effect of temperature and food availability on cellular energy budgets (via use of the cellular energy allocation (CEA) technique) and condition index; ii) the effect of temperature on oxygen consumption rates; and iii) the effects of coinciding temperature and hypoxia to condition index. Warming had an impact to both CEA and oxygen consumption rates while there was no effect of warming and/or hypoxia to condition index. Results highlight that tissue type and food availability are important considerations when assessing the effects of warming on marine bivalves, and indicate that cellular changes in energy budgets and altered oxygen consumption rates may be used as a tool to detect temperature impacts before observable changes in whole body condition occur.

5.2 Aims and Objectives

Aims:

- Examine the impacts of climate change (warming and hypoxia) to energy budgets (i.e. availability, allocation, consumption) in *M. modiolus*;

- Examine energetic responses across varying levels of organisation (i.e. cellular, whole organism) to assess the potential of various approaches for monitoring climate change impacts on *M. modiolus* reefs.

Key Objectives:

- **Investigate the effect of temperature stress to cellular energy allocation (CEA) and condition index** in *M. modiolus* with additional consideration of food availability via a short-term exposure to temperatures representative of the species' range (including upper thermal limits) under restricted and high feed rations;

- **Investigate the effect of temperature to oxygen consumption** in *M. modiolus* via quantification of oxygen consumption rates under temperatures representative of the species' range (including upper thermal limits);

- **Investigate the effect of coinciding temperature and hypoxia stress to condition index** in *M. modiolus* via a medium-term exposure to temperatures representative of the species' range (including upper thermal limits) and normoxic/hypoxic oxygen conditions.

5.3 Introduction

While some marine invertebrate species may be able to acclimate to changing conditions, the physiological mechanisms that enable extension of tolerance limits under changing conditions can be energetically demanding, and may therefore come at the expense of other critical functions including growth and reproduction (Pörtner, 2010; Sokolova, 2013). These thermal stress mechanisms are also thought to be energetically costly (Feder & Hofmann, 1999; Whiteley & Mackenzie, 2016) and changes to the available energy budget of an organism may directly impact its ability to mount an effective acclimatory or stress response (Hofmann & Todgman, 2010). Additionally, physiological and biochemical compensation for temperature change can influence thermal tolerances and species distribution patterns (Somero 2002; Peck *et al*., 2009; Tomanek, 2011).

Stress conditions create metabolic energy demands that may exceed energy supplied from food and/or energy stores of somatic tissues which may overwhelm the capacity of physiological systems and cellular mechanisms involved in maintenance of routine metabolism (e.g. ATP production) (Lannig *et al.,* 2010). Consequently, the organism may be confronted by a complex set of physiological challenges that demand a considerable adjustment to energy allocation between competing processes linked to damage-repair, growth, cell proliferation, and reproduction (Somero *et al*., 2011). This complexity of response has resulted in a poor understanding of the compromises and trade-offs that may be employed by organisms to survive in increasing stressful environments (Navarro *et al*., 2012).

Little is known about the energy budgets of subtidal bivalves such as *M. modiolus* compared to inter-tidal species (Ezgeta-Balic *et al*., 2011). The species possesses a relatively slow growth rate (Fariñas-Franco *et al.,* 2014) and tends to lack a distinct spawning window (Holt *et al.,* 1998; Roberts *et al.,* 2011) and consequently has been characterised as having relatively stable energy stores throughout the year, especially compared with well-studied species such as *M. edulis* (Navarro & Thompson, 1996). However, previous investigation shows that the effects of seasonal differences in temperature and food availability on the energy budgets of *M. modiolus* are an important consideration. For example, while *M. modiolus* has been shown to compensate for poor food availability by increasing absorption efficiency (Navarro & Thompson, 1996), scope for growth (SfG; i.e. energy balance resulting from difference between assimilated ration

and energy lost to respiration) and net growth efficiency (K2) in *M. modiolus* may be seasonally-driven with lowered metabolic rates, low temperatures and energy-rich food supply occurring during spring phytoplankton blooms and reduced SfG and K2 observed during warmer summer months (Navarro, 1990). Similarly, in milder winters, increased rates of body mass loss have been observed in bivalves due to increased metabolic demands during a time of low food supply (Honkoop & Beukema, 1997). Consequently, it is predicted that climate-induced warming will alter energy budgets in *M. modiolus* but that response will be influenced by food availability. Investigation into the impacts of other physical factors, such as ocean acidification, on *M. edulis* also suggests that food availability plays a large role in determining whether physiological processes in bivalves are influenced by a changing environment with access to food reducing the overall effect (Thomsen e*t al.,* 2012; Mackenzie *et al.,* 2014).

Stress conditions can have functional consequences at all levels of biological organisation ranging from molecular and biochemical reactions to cellular, tissue and organ function to the integration of all components at the whole-body level (Guderley & St-Pierre, 2002). Similarly, energy availability and expenditure may be detected across varying levels of biological organisation to examine the metabolic costs associated with environmental stressors from various perspectives including at the cellular level (cellular energy budgets and oxygen consumption rates) and also at the level of the whole-organism (condition index).

Cellular Energy Budgets

Few studies have determined the energetic consequences of climate change at the level of the cell where it is possible to study both energy availability and energy consumption. The cellular energy allocation technique (CEA) allows such an approach to be adopted by assessing energy reserves and energy consumption at the cellular level to determine energy budgets. The method quantifies available energy reserves (lipids, protein, and glycogen) and energy consumption (enzyme activity of the electron transport system (ETS)) at a cellular level of biological organisation and incorporates both components into a net cellular energy budget (Erk *et al.,* 2011; Wang *et al.,* 2012). CEA has chiefly been used in ecotoxicology assessments of a range of taxa, including bivalve molluscs (e.g. Smolders *et al*., 2004; Erk *et al*., 2011). More recently, CEA has proved a potentially valuable tool for detecting impacts of physical environmental stressors. Erk *et al*. (2011),

for example, reported increased energy consumption in *Mytilus galloprovincialis* under increased osmotic stress while Wang *et al.* (2012) observed a significant decline in energy reserves in scallops (*Chlamys farreri*) following exposure to increases in temperature.

Oxygen Consumption

Climate change stressors can have a profound effect on marine invertebrates by influencing rates of biological processes within defined limits (Peck *et al.* 2009; Whiteley & Mackenzie, 2016). Physiological rate processes increase with temperature over the normal temperature range of a species (Pörtner, 2010; Schulte *et al*., 2011) and marine bivalves have been shown to vary their oxygen consumption (via changes in respiratory and filter-feeding rates) to compensate for increases. Bayne *et al*. (1976), for example, observed that *M. edulis* had an acute increase in ventilation and oxygen uptake rates when first exposed to a temperature increase but could acclimate to a 5°C temperature change over a two-week period. However, *M. edulis* inhabits the intertidal and is acclimatised/adapted to variable environments while *M. modiolus* inhabits subtidal and therefore relatively stable thermal marine environments, and may be more susceptible due to having restricted physiological capacities to adjust to thermal fluctuations (Whiteley & Mackenzie, 2016).

Elevated CO_2 concentrations cause increases in standard metabolic rates (SMR) and shifts in metabolic pathways in bivalves (Beniash *et al*., 2010; Lannig *et al*., 2010). Likewise, combined exposure to thermal and acidification stressors has been observed to elevate SMRs in bivalve species, indicating an elevated cost of basal maintenance under such conditions (Mattoo *et al.*, 2013). Additionally, increases in oxygen consumption associated with increased SMRs typically result in increased production of reactive oxygen species (ROS), leading to further cell damage and increased energy demands for repair or replacement of damaged cellular components (Kültz, 2005). According to the concept of oxygen and capacity-limited thermal tolerance, an increase in temperature beyond the critical temperature (CTmax), when there is a mismatch between oxygen delivery and supply, results in the involvement of repair mechanisms for the protection and repair of molecular structures (Pörtner, 2010; Sokolova, 2013). Thus, any impact of temperature on oxygen consumption rates could suggest additional effects to *M. modiolus* with regards to oxidative stress response (refer to Chapter 4).

Condition Index

Condition Index (CI) is a recognised indicator of available energy reserves at the whole organism level of organisation. CI is frequently used as a measure of the health and commercial quality of bivalves and as an indicator of the eco-physiology of species (Orban *et al*., 2002). Changes in abiotic factors may influence the meat yield and biochemical composition of bivalves (Fernández-Reiriz *et al*., 1996), and thus CI may have value in illustrating climate change effects to the whole animal Additionally, under some stress conditions (e.g. temperature change) whole-animal responses have been observed to be more sensitive than biochemical reactions, so it may be important to include CI in an investigation to be sure that any impact is detected (Pörtner, 2010; Schulte *et al*., 2011).

In this chapter, the impacts of climate change stressors (warming, hypoxia) to CEA, oxygen consumption and CI in *M. modiolus* are examined so as to illuminate potential impacts to energy budgets under climate change conditions. Three experiments were carried out to examine each aspect. In Experiment 1 the balance between cellular energy availability and energy consumption of *M. modiolus* after acclimation to an environmentally-relevant temperature range and under changing feed availability was investigated. CEA was determined following low and high feeding periods in gill, gonad and adductor muscle from individuals held at each temperature. CI was also calculated as a measure of potential impacts at the whole-body level. Experiment 2 followed on from this by examining oxygen consumption rates in *M. modiolus* under the same temperature range in order to determine impacts to energy uptake under climate change conditions. Finally, in Experiment 3 CI was investigated following a month-long exposure to coinciding warming and hypoxia conditions. Cumulatively, results provide a comprehensive illustration of potential impacts to energetics across varying scales of organisation, and additionally, aid in assessment of different methodologies for biomonitoring purposes.

5.4 Methodology

Experiment 1. Effects of temperature to cellular energy allocation and condition index in M. modiolus under varying feed availability

Collection

Adult *M. modiolus* (mean length=107± 8.7 mm) were collected by the Heriot Watt Scientific Dive Team from 25.6m depth (temperature = 14°C) at Loch Linnhe (near Port Appin) on the west coast of Scotland (56° 33' 1.8'' N, 005° 25' 26.4'' W) in September 2014. Mussels were transported in cold boxes to the Scottish Association of Marine Sciences (SAMS) in Oban, Scotland where they were transferred to one large (~400L) holding tank, receiving a constant supply of flow-through seawater (mean seawater temperature 15°C) sand-filtered prior to delivery. Seawater algal supply was supplemented once per week with concentrated algal feed product (Shellfish Diet 1800, Reed Mariculture, Campbell, California). Animals were maintained under these conditions for approximately three months before beginning the acclimation/restricted feed experiment. Ten animals were frozen for determination of baseline condition index prior to experimentation.

Acclimation/Restricted Feed Experiment

M. modiolus were acclimated to four experimental temperatures (10°C, 13°C, 16°C, 19°C) for approximately four weeks. Twenty-three bivalves were placed into each of two flow through tanks (~50L) held at 10°C or 13°C, and an additional 46 bivalves into a flow through tank held at 16°C. After one week, 23 bivalves were moved from the 16°C tank to a tank held at 19°C. Temperatures were recorded throughout via use of loggers (Hobo Pendant Temperature Data Logger, Measurement Systems Ltd., Newbury, UK). The temperatures were chosen to cover the normal temperatures experienced by *M. modiolus* in the field (10-16°C) (Kent, 2015) as well as an additional temperature (19°C) approximating critical thermal maximum (CT_{max}) for *M. modiolus* (Halanych *et al.*, 2013) in order to study the energetic consequences of both temperature acclimation and thermal stress. Additionally, impacts to condition index were measured to provide indication of parallel effects at the level of the whole organism.

Acclimation tanks were supplemented with algal feed product (Shellfish Diet 1800, Reed Mariculture, Campbell, California) every two weeks, from here on referred to as the restricted feed protocol. At the end of the acclimation period, tissues were dissected from

five bivalves taken from each temperature treatment in order to determine cellular energy allocation (CEA). Adductor muscle, gill and gonad tissue samples (~500 mg) were dissected from each animal, immediately flash frozen in liquid nitrogen and stored at -70°C until CEA determination. These tissues were targeted due to their key roles in the physiology (respiration, filter-feeding, reproduction) and ecology (biodeposition, protection against predation) of the species. Additionally, five animals from each temperature treatment were frozen for subsequent determination of condition index.

Tissue-specific differences were investigated by studying CEA in the gill, gonad and adductor muscle of *M. modiolus* following a month-long acclimation period to four temperatures at restricted feed availability and following an additional week of high feed supply.

High Feed Experiment

For the high feed experiment, 12 large (~300L) Vortex Resuspension Tanks (VoRTs) (Davies *et al*., 2009) were utilised to keep feed (i.e. algae) in suspension and thereby ensure suitable feeding conditions for *M. modiolus* (Figure 5.1). Each VoRT (200L) received continuous supply of filtered seawater, and aquarium pumps and individual air supplies were used to create vortex suspension conditions. A randomised block design was applied to assign three VoRTs to each of the four experimental temperatures (10°C, 13°C, 16°C, 19°C). Temperatures were maintained via a system of submerged heaters and in-line chilling units for each VoRT. Temperature was monitored across all tanks for two weeks prior to the start of the high feed experiments. Temperature was recorded throughout via use of loggers (Hobo Pendant Temperature Data Logger, Measurement Systems Ltd., Newbury, UK).

Following the temperature acclimation/restricted feed period, four individual *M. modiolus* were transferred from the corresponding temperature acclimation tank and randomly assigned to each VoRT with three VoRTs per temperature treatment. Each bivalve was placed within a short piece of aquarium piping (approximately 10 cm diameter, 10 cm height) in the same orientation (i.e. siphons pointing upwards) to ensure similar feeding potential and housings were arranged randomly around the central upweller of the VoRT (Figure 5.1). A 12:12 photoperiod was applied.

Figure 5.1. Experimental set-up for determining the effect of temperature to cellular energy budgets and condition index in *M. modiolus* under conditions of high feed availability. Top image (i) shows VoRT system including tanks (t) (held at varying temperatures) and upweller apparatus (ua) and air controller (ac) for maintaining suspension of algae in each tank. Rates of algal delivery were controlled via a peristaltic pump, shown in bottom left image (ii). Bottom right image (iii) shows inside of VoRT including upweller apparatus (ua), algal delivery tube (adt) with mussels (m) situated as base of VoRT around central upweller apparatus (ua).

Bivalves were provided a constant supply of algal feed (Shellfish Diet 1800, Reed Mariculture, Campbell, California). Concentrated algal feed was diluted in seawater and distributed to all VoRTs via peristaltic pumps (205S/CA manual control 12-channel cassette pump, Watson-Marlow, Cornwall, UK) (Figure 5.1). The target food concentration was 7 µg chlorophyll a l^{-1} or 3.9 mg total particulate matter l^{-1} based on field measurements completed by Kent (2015), from here on referred to as the high feed protocol. Food concentration was chosen to represent the natural food available in Loch Linnhe in summer months (Kent, 2015), therefore representative of high food availability.

Following one week, the tissues from two animals from each VoRT were dissected for CEA determination resulting in six individuals per temperature treatment. Gill, gonad and adductor muscle samples (~500 mg) were dissected from each animal, flash frozen in liquid nitrogen and stored at -70°C before processing. The remaining bivalves were used to determine condition index of each temperature treatment. Shell length and sex were recorded for each animal at the time of dissection.

Condition Index

All condition index samples were defrosted over 12 hours. Soft tissues from each animal were dissected into pre-weighed metal weigh boats and tissues were oven-dried at approximately 60°C for 72 hours. Following, dry weights of tissues were determined (Sartorius Analytic, Surrey, UK). All shell lengths were taken to the nearest millimetre using Vernier callipers. Condition index (CI) of each mussel was determined as $CI=DW_f / SL^3$ where DW_f=mg total soft tissue dry weight and SL=shell length (cm) according to Clausen & Riisgard (1996).

CEA Determination

The cellular energy allocation (CEA) method was based on the biochemical assessment of changes in cellular energy reserves and cellular energy consumption (De Coen & Janssen, 1997). Energy reserves were determined by measuring the total lipid, carbohydrate and protein content of the various tissues. The energy consumed was estimated by measuring the electron transport activity (ETS) at the level of the mitochondria (Erk *et al.,* 2011).

Protein Content

Protein content was measured using the microplate version of the Pierce BCA Protein Assay Kit (Thermo Scientific). Approximately 20 mg of frozen tissue sample (gill, gonad or adductor) was homogenised in 0.1M Tris-HCl buffer (pH 6.8) and centrifuged at 3 000g for 10 min, 4°C. The resulting supernatant was added in triplicate to a well-plate before addition of Bradford's solution (Bradford, 1976). Absorbance was read at 385 nm (Multiskan FC, Thermo Scientific) using a protein standard (Bovine Standard Albumin, Sigma-Aldrich).

Carbohydrate Content

Carbohydrate content was determined using the anthrone-sulphuric acid assay (Roe & Daily, 1966; Smolders *et al.,* 2004). Approximately 15 mg of frozen tissue sample (gill, gonad or adductor) was homogenised in 15% Trichloroacetic acid (TCA). Following homogenisation, all samples were placed on ice to precipitate proteins. Samples were then centrifuged (3 000 g for 10 min, 4°C) and ice cold 0.2% anthrone-sulphuric acid solution was added to the resulting supernatant before samples were heated to 95°C for 5 minutes. After cooling, samples were added in triplicate to a well-plate and absorbance was read at 620 nm (Multiskan FC, Thermo Scientific) using glycogen as a standard (ml^{-1}).

Lipid Content

Total lipid content was determined using the sulphophospho-vanillin method according to Zöllner & Kirsch (1962) modified for microplates by Torres *et al.* (2007). Approximately 20 mg of frozen tissue sample (gill, gonad or adductor) was homogenised in distilled water. Chloroform-methanol (2:1) solution was added to the homogenate prior to incubation at room temperature (15 min) and centrifugation (10 000 g for 20 min, 4°C). The lower chloroform phase was transferred to glass tubes and incubated (60°C) for approximately 40 min. After cooling, concentrated sulphuric acid was added to all samples before incubation (95°C) for 10 min. After the addition of 8 mM phospho-vanillin solution, samples were incubated at room temperature for 40 minutes in the dark. Absorbance was read at wavelength of 530 nm in a plate reader (Multiskan FC, Thermo Scientific) using cholesterol as a standard.

Energy Consumption

Energy consumption was measured as electron transport activity (ETS) according to Owens & King (1975), and modified for microplates by Smolders *et al*. (2004). Approximately 20 mg of frozen sample (gill, gonad or adductor) was homogenised in Tris-HCl buffer (pH 8.5). All samples were centrifuged (3 000 rpm for 10 min, 4°C) and the resulting supernatant added in triplicate to a well-plate. Buffered substrate solution was then added to supernatant and the reaction was initiated via addition of *p*-IodoNitroTetrazolium (INT). Absorbance was immediately measured every 30 seconds for 10 minutes at a wavelength of 490 nm in a plate reader (Multiskan FC, Thermo Scientific).

CEA

All energy fractions (protein, carbohydrate, lipid) were converted to energy equivalence units via combustion values determined by Gnaiger (1983) (glycogen: 17 500 mJ mg^{-1}; protein: 24 000 mJ mg^{-1}; lipid: 39 500 mJ mg^{-1}) and fractions determined in mJ mg^{-1} wet weight. The sum of energy fractions provided the total energy available (Ea) for each tissue type. Energy consumed (Ec) was determined in mJ mg^{-1} wet weight $hour^{-1}$ from the ETS data and CEA was calculated as Ea/Ec (Erk *et al*., 2011).

Analyses

Data were tested for normality and homogeneity of variances (Levene's Test). If necessary, data were log-transformed to meet assumptions. Two-way ANOVAs were carried out to determine the effect of temperature and feeding regime. Where a factor was found to cause a significant effect, post-hoc (Tukey HSD) tests were carried out to identify specific differences. Where a significant interaction was found, one-way ANOVAs (with post-hoc (Tukey HSD) for temperature comparison) were carried out to investigate the effect of temperature to each feeding regime and to determine the effect of feeding regime at each temperature. Additionally, for analyses of gonad tissue, a two-way ANCOVA was applied with sex as a covariate. Data are presented as means ±1 SE unless otherwise stated. Statistical analyses were performed using SPSS software (SPSS 14, SPSS INC, Chicago, IL, USA). All reported significance levels are $p<0.05$.

Experiment 2. Effects of warming to oxygen consumption in *M. modiolus*

Collection

Adult *M. modiolus* (mean length=103± 9.2mm) were collected by divers from 24m depth at Port Appin on the west coast of Scotland (56°32.897'N; 05°17.754'W) in January 2016. Mussels were transported in cold boxes to Heriot-Watt University (Edinburgh, Scotland) where they were transferred to one large (~500L) holding tank where they received a constant supply of filtered seawater via a re-circulation aquarium system. Animals were maintained at approximately 14°C for five weeks prior to experimentation and fed *ad libitum* with *Tetraselmis suecica* three times per week.

Experimental Set-up

Oxygen consumption rates in *M. modiolus* were measured under four different temperature treatments: 10°C, 13°C, 16°C, 19°C. Temperatures were chosen to duplicate conditions as per Experiment 1, and were representative of the normal temperatures experienced by *M. modiolus* in the field (10-16°C) (Kent, 2015) as well as the approximate CT_{max} (19°C) for the species. *M. modiolus* (n=6) were placed in 500 mL airtight chambers pre-filled with filtered seawater (at experimental temperature) and fitted with stirrers controlled by an external magnetic stirrer (Figure 5.2). All chambers were then placed into a water bath maintained at the experimental temperature via a bar heater (Titanium 300KW heater, Aquamedic, Coalville, UK) and submersible pump (Compact 600, Eheim Deizisau, Germany). Animals were acclimated to each experimental temperature for three days prior to experiment start. They were not fed during the acclimation period as per Hennige *et al*. (2015).

Oxygen Consumption Rates

Oxygen levels (%) were measured via an oxygen optode system (Oxy-4 with Temp-4, Presens & Loligo systems) over a four-hour period (Figure 5.2). In brief, an oxygen fibre optic probe was inserted into a small cavity in the lid of each chamber with a chemical sensor dot mounted at its base (in direct contact with seawater). Sensor spots were calibrated against air-saturated water and oxygen-free water. A fluorescence reading was taken every 15 seconds at which point the optode would illuminate the sensor dot with a blue LED. The blue LED excited the sensor pad which then re-emitted a red luminescent light with strength dependent on level of oxygen present. A stronger signal was re-emitted when fewer oxygen molecules were present (as oxygen molecules quench the

signal) and thus, the level of oxygen was determined according to the strength of re-emitted red light (Loligosystems Instruction Manual). Each chamber was fitted with its own optode and oxygen measurements (%) were automatically adjusted to experimental temperatures via the Loligo software. The first 30 minutes of each reading were dismissed to give animals time to adjust to conditions and begin filtering. An additional empty control chamber was run alongside the other chambers to monitor and measure microbial respiration. Animals were kept in a lit room during measurements but light was diffused via dark plastic sheeting (placed over chambers) to mimic natural conditions. Animals were not fed during the experiment due to constraints of experimental set-up (i.e. closed sealed chambers). All measurements were carried out within a one week period. When a set of measurements was complete, each individual mussel was removed from its chamber and dissected to determine wet weight (g). Additionally, the volume of seawater in each chamber was measured.

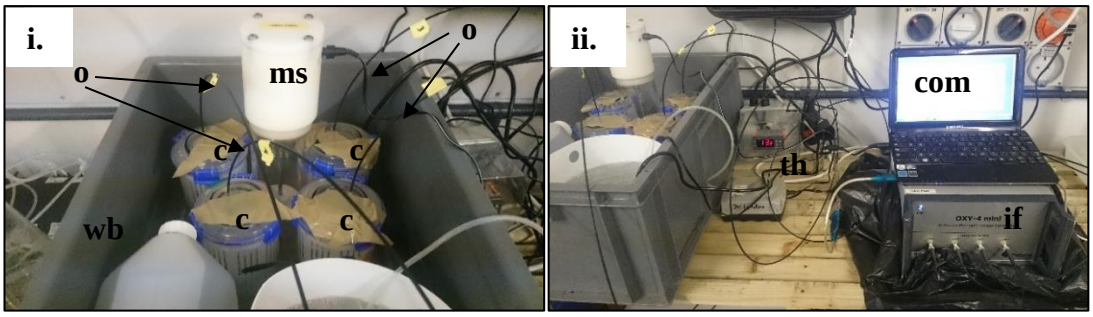


Figure 5.2. Experimental set-up for determining the effect of temperature to *M. modiolus* oxygen consumption rates. Left image (i) shows water bath (wb) with chambers (c) with optodes (o) inserted into chamber lids, and situated around magnetic stirrer (ms). Right image (ii) shows entire set-up including Oxy-4 interface (if) and linking computer (com) for recording oxygen levels over time and thermostat (th) for maintaining and monitoring experimental temperatures.

Analyses

Oxygen consumption rates were determined in mmol O_2 gram wet weight^{-1} hour^{-1}. One-way ANOVA was used to compare rates between temperature treatments. Data were tested for assumptions of normality and homogeneity of variances (Levene's Test). Where data failed to meet assumptions, log or square-root transformations were applied. All statistical analysis was carried out on SPSS 14.0 for Windows (2005).

Experiment 3. Effects of warming and hypoxia to condition index in M. modiolus

Collection and Experimental Set-up

Condition index was measured in the same animals as were used for oxidative stress experiments (refer to Chapter 4 pp. 150-155 for full details of collection and experimental set-up). In brief, *M. modiolus* from Karlsruhe (Orkney, Scotland) and North Lleyn (Wales) were held at 10°C, 14°C or 18°C for four weeks in a flow-through aquarium system at St. Abbs Marine Station. Temperatures were maintained via a system of water baths attached to inline cooling and heating units with additional bar heaters for high temperature treatments. For the final week, animals were assigned to either normoxic (~8mg O_2 L^{-1}) or hypoxic (~2mg O_2 L^{-1}) oxygen conditions to examine co-stressor effects of warming and hypoxia. Hypoxia levels were determined according to levels reported by Vaquer-Sunyer & Duarte (2008). Hypoxia conditions were achieved via bubbling of nitrogen gas into seawater until 2mg O_2 L^{-1} seawater was reached and buckets were then sealed to maintain experimental O_2 levels. Daily water changes were completed to maintain experimental O_2 levels across seven days. O_2 levels were monitored daily via a hand-held temperature-salinity-dissolved oxygen meter (YSI ProDSS). Identical experimental procedures were carried out for Orkney animals (in March-April 2015) followed by Wales animals (June 2015).

Condition Index

Prior to and following the four-week exposure, baseline individuals (n=5) and *M. modiolus* from each treatment (n=5) were frozen for determination of condition index. Animals were stored at -20°C until determination of condition index. Samples were then thawed over 24 hours, and wet tissues dissected out and oven dried for three days at 55°C. Shell lengths were taken using callipers (nearest 0.1mm). Condition index (CI) of each mussel was determined as $CI=DW_f / SL^3$ where DW_f=mg total soft tissue dry weight and SL=shell length (cm) according to Clausen & Riisgard (1996).

Analyses

Two-way ANOVA was used to test the effect of temperature and/or oxygen level to condition index. Data were tested for assumptions of normality and homogeneity of variances (Levene's Test). Where data failed to meet assumptions, log or square-root transformations were applied. All statistical analysis was carried out on SPSS 14.0 for Windows (2005).

5.5 Results

Experiment 1. Effects of temperature and changing feed availability to cellular energy allocation and condition index in M. modiolus

CEA

Gill Tissue

All CEA values for gill tissue are provided in Appendix B, Table B6. There was a significant positive influence of temperature to energy availability (Ea) (ANOVA, $F_{(3,40)}$=7.002, P=0.001) and energy consumption (Ec) (ANOVA, $F_{(3,40)}$=60.448, p<0.001) in the gill across both ration groups (combined), paralleled by a significant negative effect to CEA (ANOVA, $F_{(3,40}$=57.160, p<0.001) (Figure 5.3). Post hoc tests identified significant differences in Ea between the lowest temperature (10°C) groups and both 16°C (p<0.001) and 19°C (p=0.029) groups; in Ec between the highest temperature (19°C) group and all other temperature groups (p<0.001 for all comparisons); and in CEA between the highest temperature (19°C) group and all other temperature groups (p<0.001 for all comparisons).

Energy availability (Ea) (ANOVA, $F_{(3,40)}$=5.539, P=0.004), energy consumption (Ec) (ANOVA, $F_{(3,40)}$=64.194, p<0.001) and CEA (ANOVA, $F_{(3,40)}$=48.445, p<0.001) in the gill were influenced by a significant interaction between acclimation temperature and food ration.

One-way ANOVA of the effect of temperature at each ration level indicated that under restricted feed conditions, Ea was significantly higher in the gill at 16°C than at 10°C (ANOVA, $F_{(3,16)}$=5.916, p=0.004); Ec was significantly higher in the gill at 19°C than any lower temperature (ANOVA, $F_{(3,16)=}$=39.396, p<0.001 for 10°C, 13°C, 16°C); and CEA was significantly lower in the gill at 19°C than at any other temperature (ANOVA, $F_{(3,16)=}$=9.515, p=0.004, 0.010, 0.001 for 10°C, 13°C, 16°C, respectively). The increase in Ea in the gill observed with an increase in acclimation temperature was associated with a significant increase in lipid energy content between 10°C and 16°C (ANOVA, $F_{(3,16)}$=6.976, p=0.003). Comparison across all temperatures under conditions of high food ration revealed that CEA in the gill was the only value to change significantly, with values lower at 10°C than at 13°C (ANOVA, $F_{(3,20)}$=4.926, p=0.025) or 16°C (ANOVA, $F_{(3,20)}$=4.926, p=0.025).

One-way ANOVA of the effect of ration at each temperature indicated that under 10°C conditions, high food availability resulted in a significant increase in Ea (ANOVA, $F_{(1,10}$=21.214, p=0.002) and Ec (ANOVA, $F_{(1,10}$=144,274, p<0.001) but a significant decrease in CEA (ANOVA, F=17.016, p=0.003) in the gill. At both 13°C and 16°C, high food availability caused a significant increase in Ec in the gill (ANOVA, $F_{(1,10)}$=43.032,<0.001 and $F_{(1,10)}$=38.525, p<0.001, respectively) with parallel significant decreases in CEA (ANOVA, $F_{(1,10}$=41.229, p<0.001 and $F_{(1,10)}$=91.309, p<0.001, respectively). This decrease in CEA was associated with a significant decline in lipid reserves at both temperatures (ANOVA, $F_{(1,10}$=5.518, p=0.047 and $F_{(1,10)}$=20.544, p=0.001). At the highest acclimation temperature of 19°C, high food availability also resulted in a significant reduction in lipid reserves (ANOVA, $F_{(1,10}$=48.001, p<0.001) but had no effect on any of the other measured variables.

Gonad Tissue

All CEA values for gonad tissue are provided in Appendix B, Table B6. Energy availability (Ea) (ANOVA, $F_{(3,41)}$=3.267, p=0.033), CEA (ANOVA, $F_{(3,41)}$=3.207, p=0.035), lipid energy content (ANOVA, $F_{(3,41)}$=3.148, p=0.037) and carbohydrate energy content (ANOVA, $F_{(3,41)}$=3.877, p=0.017) in the gonad were influenced by a significant interaction between acclimation temperature and food ration (Figure 5.4).

One-way ANOVA of the effect of temperature at each ration level showed that under restricted feed conditions, carbohydrate content was significantly higher at 13°C than 10°C (ANOVA, $F_{(3,16)}$=5.078, p=0.012) and 19°C (ANOVA, $F_{(3,16)}$=5.078, p=0.022) while under high food ration, lipid content was significantly higher at 16°C than 19°C (ANOVA, $F_{(1,10)}$=5.632, p=0.042).

One-way ANOVA of the effect of ration at each temperature showed that under 13°C conditions, high feed conditions caused a significant decrease in carbohydrate energy content (ANOVA, $F_{(1,10)}$=11.296, p=0.008).

When sex was considered as a co-factor, there was no significant effect of temperature or high feed availability but a significant interaction of the two factors for carbohydrate (ANCOVA, $F_{(3,41)=}$4.389, P =0.010) and protein content (ANCOVA, $F_{(3,41)}$=4.952, p=0.006).

Adductor Muscle

All CEA values for adductor muscle tissue are provided in Appendix B, Table B6. Across all groups, temperature had a significant negative effect on CEA in the adductor muscle (ANOVA, $F_{(3,41)}$=3.035, p=0.042) with post hoc tests indicating increased CEA at 10°C as compared to 19°C (p<0.040). There was no significant interaction between acclimation temperature and food ration (Figure 5.5, Table 5.1).

Condition Index

Mean condition index in *M. modiolus* prior to temperature acclimation was 5.38 ± 0.34, *n* = 10. Following restricted feed for four weeks at 10°C, 13°C, 16°C, and 19°C, condition indices were 4.46 ± 0.39, *n* = 5; 4.52 ± 0.41, *n* = 5, 5.21 ± 0.38, *n* = 5, 5.34 ± 0.47, *n* = 5, respectively, and following high feed were 4.68 ± 0.87, *n* = 6; 5.83 ± 0.58, *n* = 6; 4.94 ± 0.91, n = 6; and 4.58 ± 0.20, *n* = 6, respectively (Figure 5.6). Two-way ANOVA showed that neither temperature nor feed ration influenced condition index and there was additionally no interaction between the two factors.

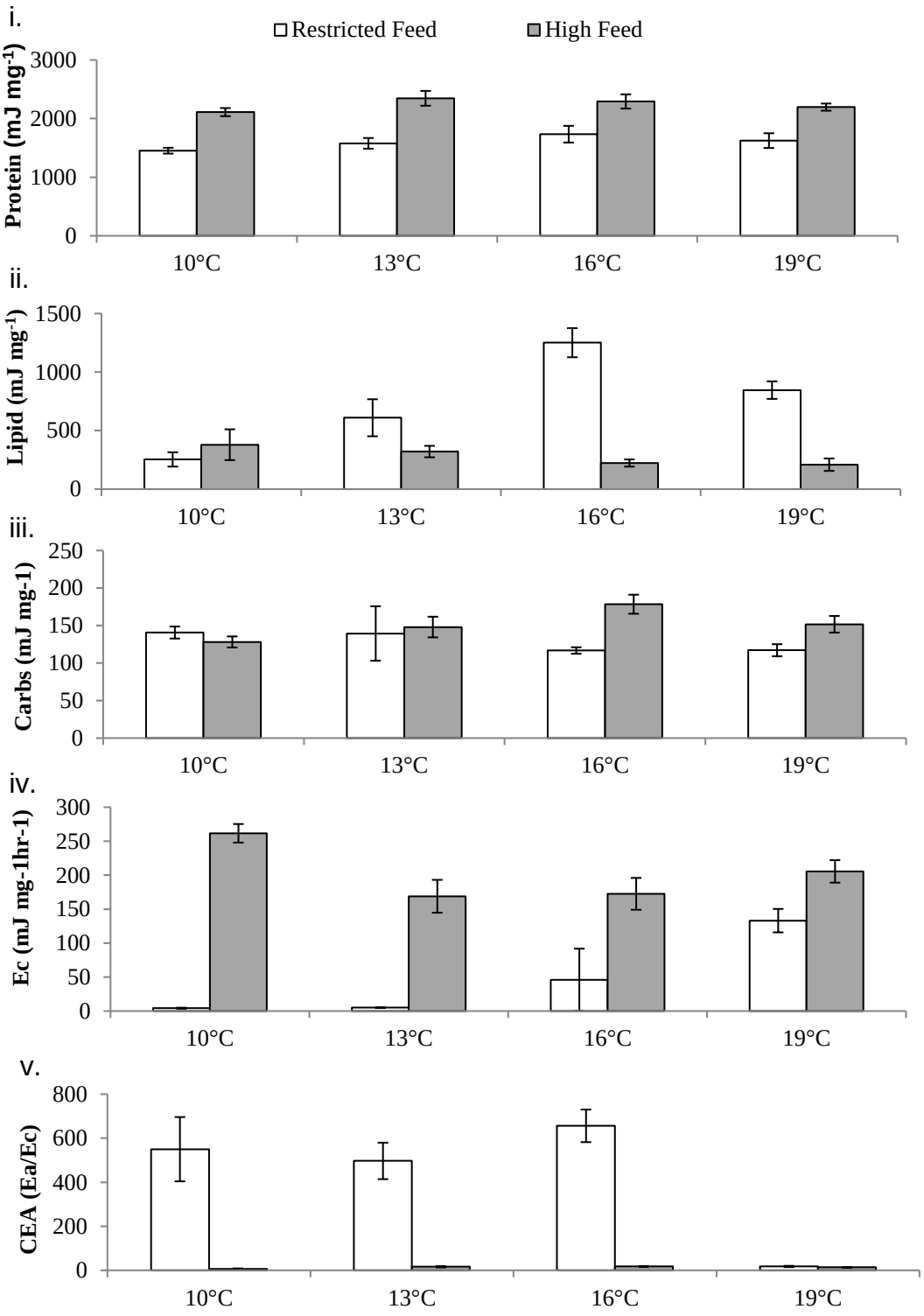


Figure 5.3. (i) Protein, (ii) lipid and (iii) carbohydrate (carb) cellular energy stores (mJ mg^{-1} wet weight), (iv) energy consumption rate (Ec) (mJ mg^{-1} wet weight hr^{-1}) and (v) CEA in **gill tissue** of *M. modiolus* following restricted feed (white bars) and high feed (grey bars) periods at four acclimation temperatures (10°C, 13°C, 16°C, 19°C). Error bars represent±1SE (n=5 for restricted feed; n=6 for high feed).

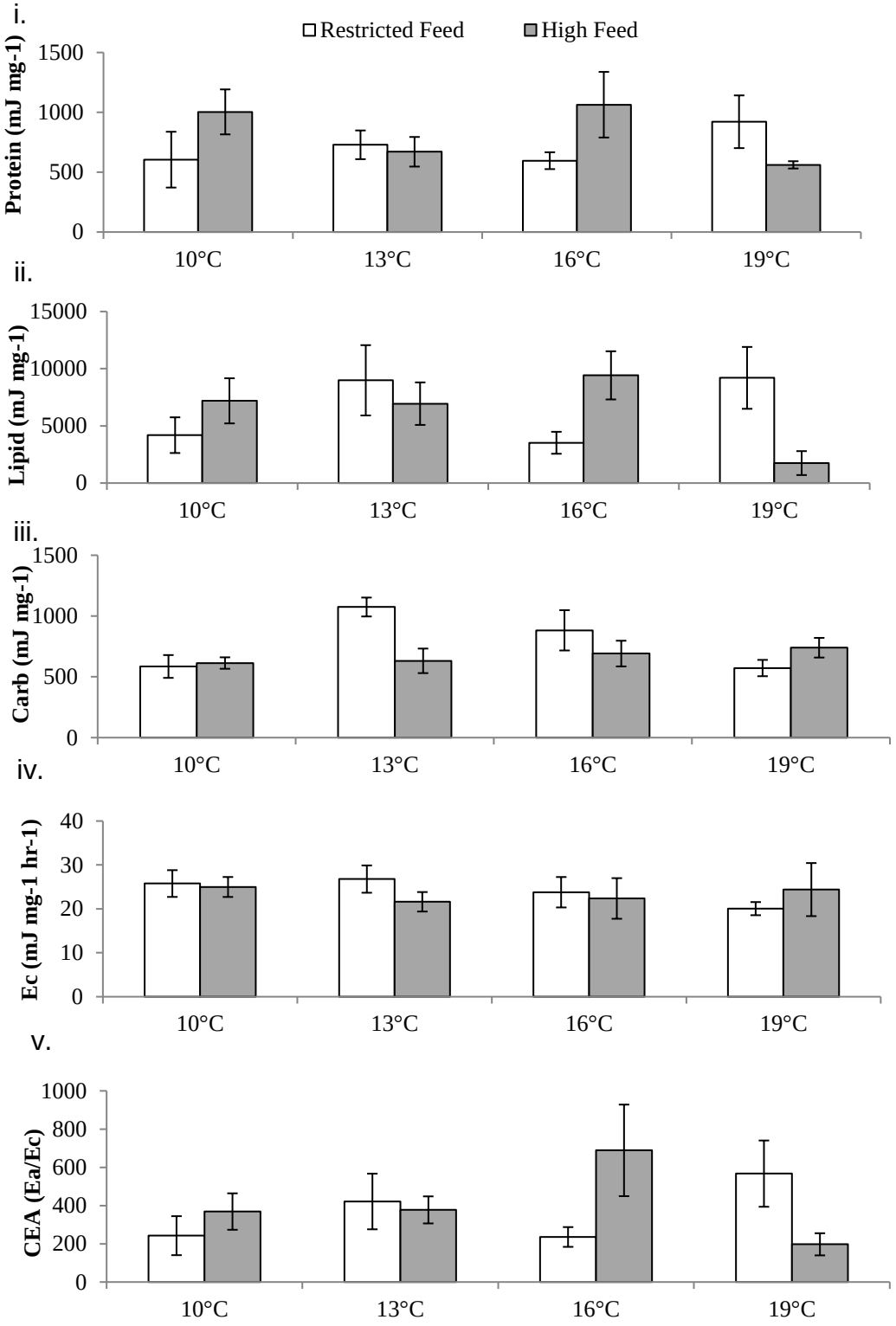


Figure 5.4. i) Protein, (ii) lipid and (iii) carbohydrate (carb) cellular energy stores (mJ mg^{-1} wet weight), (iv) energy consumption rate (Ec) (mJ mg^{-1} wet weight hr^{-1}) and (v) CEA in **gonad tissue** of *M. modiolus* following restricted feed (white bars) and high feed (grey bars) periods at four acclimation temperatures (10°C, 13°C, 16°C, 19°C). Error bars represent±1SE (n=5 for restricted feed; n=6 for high feed).

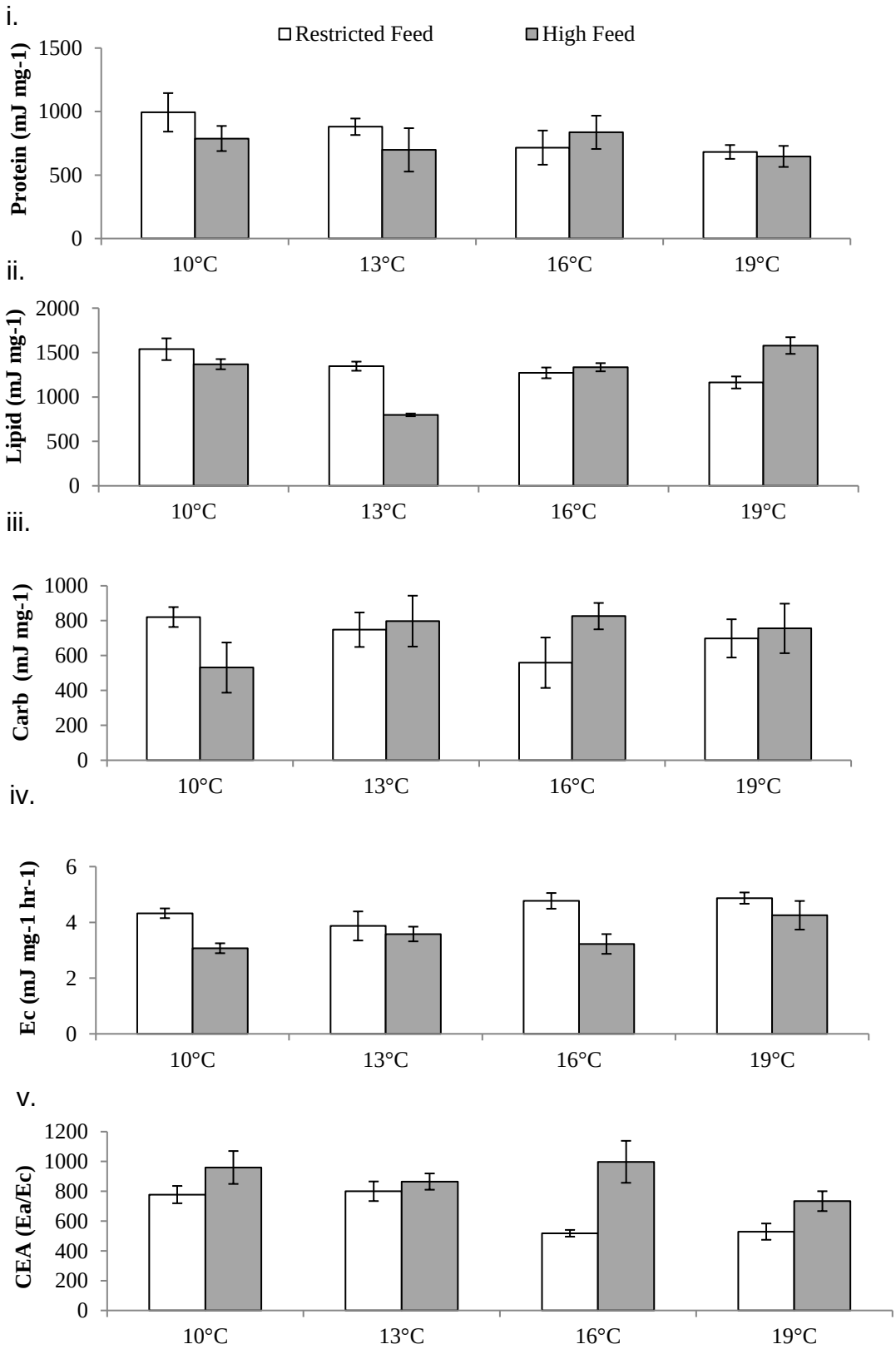


Figure 5.5. i) Protein, (ii) lipid and (iii) carbohydrate (carb) cellular energy stores (mJ mg^{-1} wet weight), (iv) energy consumption rate (Ec) (mJ mg^{-1} wet weight hr^{-1}) and (v) CEA in **adductor muscle** of *M. modiolus* following restricted feed (white bars) and high feed (grey bars) periods at four acclimation temperatures (10°C, 13°C, 16°C, 19°C). Error bars represent±1SE (n=5 for restricted feed; n=6 for high feed).

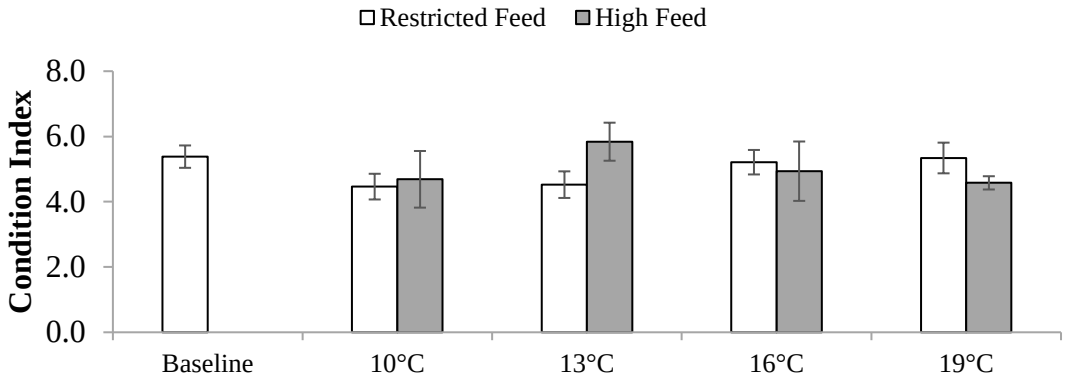


Figure 5.6. Condition index in *M. modiolus* at baseline (dark grey bar) and following restricted feed (white bars) and high feed (light grey bars) periods at four acclimation temperatures (10°C, 13°C, 16°C, 19°C). Error bars represent±1SE (n = 10 for baseline; n=5 for restricted feed; n=6 for high feed).

Experiment 2. Effects of warming to oxygen consumption rates in M. modiolus

Consumption rates were square root transformed to meet parametric assumption of homogeneity of variance. There was a significant effect of temperature to oxygen consumption rates of *M. modiolus* ($F_{(3,17)}$=17.331, p<0.001). Oxygen consumption rates in animals held at 13°C were significantly higher than 10°C (p=0.003) or 16°C (p=0.001). Likewise, animals held at 19°C were significantly higher than 10°C (p=0.001) or 16°C (p<0.001) (Figure 5.7).

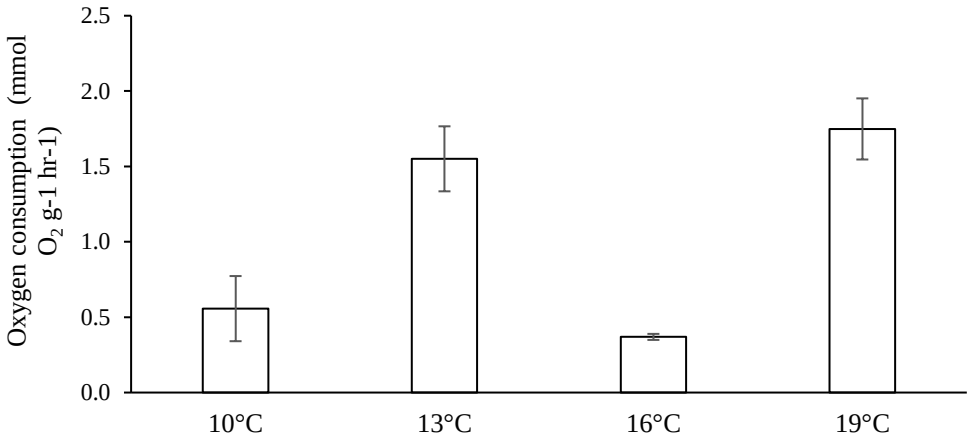


Figure 5.7. Oxygen consumption rates (mmol O_2 g^{-1} hr^{-1}) in *M. modiolus* held at 10°C, 13°C, 16°C and 19°C. Error bars represent±1SE (n=5 for 10°C, 13C°, 16°C; n=6 for 19°C).

Experiment 3. Effects of warming and hypoxia to condition index in M. modiolus

There was neither effect of temperature ($F_{(2,27)}$=1.8202, p=0.184) nor hypoxia ($F_{(1,28)}$=1.394, p=0.249) to condition index of Wales *M. modiolus* nor interaction between the two factors ($F_{(2,27)}$=0.587, p=0.564) Likewise, there was neither effect of temperature ($F_{(2,27)}$=0.022, p=0.978) nor hypoxia ($F_{(1,28)}$=0.045, p=0.833) to condition index of Orkney *M. modiolus* nor interaction between the two factors ($F_{(2,27)}$=1.087, p=0.353) (Figure 5.8). Comparison of populations at each treatment level also showed no differences in condition index.

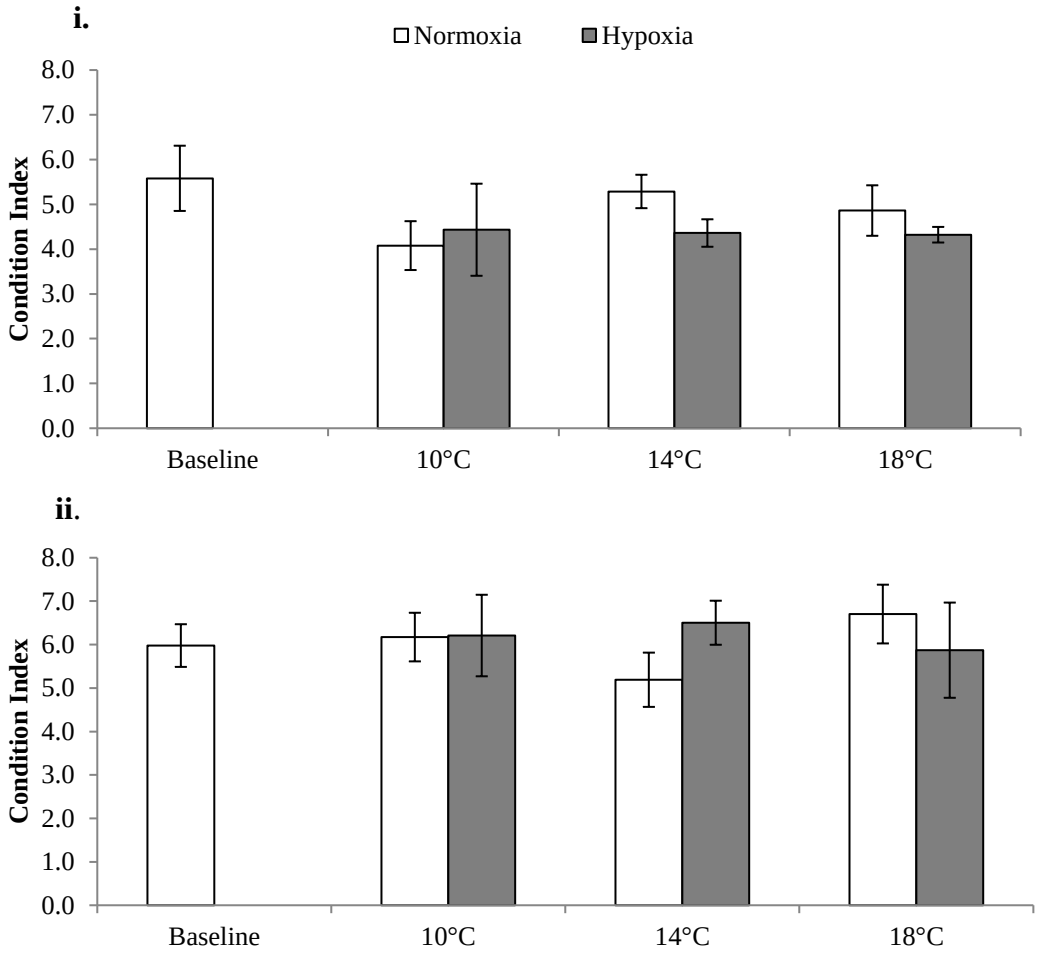


Figure 5.8. Condition index of (i) Wales, and (ii) Orkney *M. modiolus* at baseline (white bars) and following four-week exposure to 10°C, 14°C or 18°C with final week of normoxia (light grey bars) or hypoxia (dark grey bars). Error bars represent±1SE (n=5).

5.6 Discussion

Results suggest that energy budgets of *M. modiolus* may be altered under climate change (warming and/or hypoxia) conditions. However, findings indicate that response is complex and dependent on factors such as acclimatory ability and feed availability. Results also emphasise the importance of studying the effects of stressors across levels of organisation as cellular reductions in energy budgets and changes in oxygen consumption rates were observed despite no change in whole body condition.

Cellular Energy Budgets

In this investigation, initial findings are provided regarding use of the CEA method for examining cellular-level impacts of temperature to energy budgets in a sub-tidal marine ectotherm species, with additional consideration of the influence of feed ration to temperature effects. In general, gill tissue appears to be more sensitive to temperature than either gonad or adductor muscle with significant changes in energy budgets with change in acclimation temperature. However, temperature effects were strongly influenced by feed ration with high feed availability contributing to decreases in cellular energy budgets. This study highlights that both tissue type and food availability are important considerations when assessing the effects of warming on marine bivalves.

Gill Tissue

Increased temperature had a clear effect to cellular energy budgets of gill tissue but effect varied considerably according to availability of food (i.e. restricted feed ration vs. high feed ration). Following, the influence of restricted feed and high feed rations to warming impacts in gill tissue are considered separately.

Warming + restricted feed ration

M. modiolus maintained relatively high and equivalent CEA in the gill across temperatures of 10°C to 16°C, despite limited food supply. Bivalves have been observed to have extended periods of valve closure with parallel decreases in oxygen consumption as a means of conserving energy during limited feed conditions (Wang & Widdows, 1993; Riisgard *et al.,* 2003). Further, *M. modiolus* often inhabits environments that have intermittent or inadequate food supply and the species has been observed to make physiological compensations to deal with such conditions (Navarro & Thompson, 1996). For example, *M. modiolus* has demonstrated intermittent feeding behaviour as a means

of conserving energy when feed is of low nutritive value (Navarro & Thompson, 1996). Consequently, the maintenance of CEA across temperatures under low feed conditions may be an optimised response by *M. modiolus* to poor food supply. However, at a temperature approaching CT_{max} (19°C) for *M. modiolus* and under restricted feed conditions, there was an increase in cellular energy consumption and parallel decreases in CEA in the gill. Such increases in energy demands and corresponding decreases in CEA were largely expected given the physiological demands placed on ectotherm species as they move into thermal maxima (for reviews see: Pörtner, 2001; Lesser, 2006; Somero, 2010, particularly if those demands are not supported by the necessary energy supply. For example, at high temperature extremes, cellular energy requirements of thermally-induced increases in oxygen demand are amplified by increased rates of mitochondrial proton leakage, the latter heavily influencing standard metabolic rates (Pörtner, 2001).

Lipid energy stores of the gill tissue increased under increasing temperature up to 16°C with a subsequent drop in lipid energy stores in animals held at 19°C under low feed ration. The observed increase up to 16°C is surprising as increased lipid energy stores are characteristic of well-fed organisms, and typically utilised by bivalve species during periods of starvation (Thompson *et al.*, 1974; Lawrence, 1976; Gabbott, 1976). Likewise, lipids tend to play a small but nevertheless important role as maintenance energy reserves for bivalves during food-limited winter periods (Ahn *et al., 2000*) so one might have expected to see lipid energy decline in at least the lower temperature groups. Observed increases are potentially indicative of an increase in energy stores during low food conditions due to utilisation of other energy stores (e.g. glycogen) until 19°C which may indicate an upper temperature limit where energy budgets become constrained.

The decrease in lipid energy stores at 19°C (paralleled by a decrease in CEA) likely indicates that lipid stores are utilised to support energy demands under thermal stress. While bivalve species typically use glycogen over lipid reserves to meet energy requirements when faced with thermal challenges (Gabbott & Bayne, 1973; Wang *et al.*, 2012), lipids have been shown to be the main source of energy production in growing mussels during summer months (i.e. warming conditions) (Zandee *et al.*, 1980). Additionally, short-term (i.e. over hours) decreases in glucose and glycogen stores in ectotherm species under starvation stress have been observed to be followed by later decreases in lipid energy stores (Sánchez-Paz *et al.*, 2007). Thus, observed decreases in

lipids could be a consequence of both the increased temperature as well as the extended exposure time under limited food conditions.

M. modiolus has been shown to compensate physiologically for poor food availability during autumn and winter (i.e. low temperatures) by reducing filtering rates (Navarro & Thompson, 1996). However, if seasonal patterns of low feed availability are coupled with increasing temperatures (e.g. under climate change), a mismatch between food requirement (i.e. energy needs) and food availability could lead to lowered survival and recruitment (Durant *et al*., 2007). Subsequently, findings here could be particularly relevant for *M. modiolus* populations at the southernmost distribution of the species where increased metabolism under increased autumn/winter temperatures may not be supported by the necessary food requirements (Dinesen & Morton, 2014).

Warming + high feed ration

Surprisingly, between 13°C and 19°C, increased food availability contributed to a reduced energetic status in gill tissue as shown by increased energy consumption, increased utilisation of lipid energy stores, and parallel decreases in CEA. Such changes in the energetics of the gill under warming conditions coupled with high feed availability may be a consequence of the organ's direct role in both food acquisition (i.e. filter-feeding) and oxygen uptake (Bayne *et al*., 1976) and highlight the role of both factors in shaping *M. modiolus* energy budgets. Filter-feeding can be energetically costly for bivalves and animals need to balance energy gains made from feeding and ingestion against metabolic losses brought on by such activities in order to optimise distribution of surplus energy to growth and reproduction (Bayne & Newell, 1983). For example, when fed above maintenance requirements during acclimation to warm temperature (15°C), the energy budget of *M. edulis* has been observed to move into disequilibrium, representing a physiological stress potentially brought on by elevated blood sugar levels and therefore increased mobilisation and utilisation of energy reserves (Widdows & Bayne, 1971). Wang *et al*. (2012) also observed a significant decline in CEA in bivalve gill tissue under thermal stress conditions following excess ration feeding conditions.

Both temperature and feed availability directly influence seawater pumping rates in bivalves with direct consequences for oxygen consumption and therefore energy demands. Pumping rates are primarily dictated by the level of feed present in the

surrounding environment, but also rise with increasing temperature as a result of both physical (e.g. decrease in kinematic viscosity of seawater) and biological (e.g. exponential increase in metabolic activity) effects under elevated temperatures (Riisgard & Larsen, 1995). Similarly, rates of oxygen uptake have been shown to be higher in bivalves during summer months than winter (Van Winkle, 1968) due to temperature-related increases in both rates of metabolism and feeding (Bayne *et al.,* 1976). Consequently, increases in feeding rates and oxygen consumption, although not measured here, could account for the significant increase in Ec observed across all temperatures as a result of high food availability. Coinciding high temperature and high food availability could produce a negative feedback loop in *M. modiolus* where increased pumping rates (driven by high food availability) secure food for energy needs but the maintenance of such activity under high temperature causes higher energetic costs, thus highlighting a potential cost-benefit imbalance of energy acquisition under increased temperatures.

Increased feed availability caused clear changes in gill energy reserves with significant increases in protein stores and dramatic decreases in lipid stores across all temperature treatments, and increases in carbohydrate energy stores under higher temperature treatments (16°C and 19°C). Lipids and carbohydrates typically fuel normal aerobic metabolism in marine invertebrates, whereas depletion of proteins is characteristic of extreme energy deficiency (e.g. starvation) (Sokolova *et al.,* 2012). Thus, the increase in protein stores under high feed may indicate a replenishing of those stores consumed under low feed conditions. Similarly, a strong positive correlation between gill protein levels and chlorophyll a has been reported for bivalve species (Li *et al*., 2009a) and prior studies also show that protein content of *M. modiolus* tissue is highest during the spring and summer as associated with algal quality (i.e. high protein content) (Navarro, 1990; Lesser *et al.,* 1994). While bivalves have been observed to have increased energy content in storage components (i.e. carbohydrates and lipids) in early summer months, likely due to increased food availability brought on by spring algal blooms (Navarro & Thompson, 1996; Erk *et al*., 2011), the observed decreases in lipids and carbohydrate stores and parallel increase in protein stores may be indicative of energy transfer from storage components to support increased rates of filter-feeding. The dramatic fall in lipid levels in the gills at high food availability suggests that increases in Ec are fuelled by lipid utilisation preferentially over proteins and carbohydrates. A similar pattern of energy

consumption under increasing temperature has been reported for other bivalve species (e.g. *Mytilus galloprovincialis*) (Erk *et al*., 2011).

Seasonal comparison of SfG in *M. modiolus* indicates that higher SfG is associated with high concentration (and quality) of feed and reduced oxygen consumption under lower temperatures (Navarro & Thompson, 1996) and there is little data available to suggest any acclimatory ability to maintain SfG during warming. Thus, the decline in CEA observed here could suggest that increased feed availability during exposure to higher temperatures has negative repercussions for SfG and could indicate that *M. modiolus* has limited acclimatory ability as compared to *M. edulis*, but further investigation is needed.

CEA was significantly lower under the lowest temperature conditions as compared to intermediate temperatures (13°C and 16°C) when *M. modiolus* were provided high feed availability. This is unexpected as previous investigation of *M. modiolus* energy acquisition found the highest acquisition rates, scope for growth and growth rates to occur during the spring phytoplankton bloom (i.e. conditions of low temperatures and high feed availability) (Navarro & Thompson, 1996).

Gonad Tissue

There was no obvious effect of temperature on energy stores, cellular energy consumption or CEA in gonad tissue, regardless of feed condition. Other bivalve species (e.g. *M. edulis*) have also been observed to be capable of continued gonad development even during low food conditions and loss of energy reserves (Gabbott, 1976). However, given the relative lack of (or inconsistency in) understanding regarding the reproductive cycle and larval development of *M. modiolus*, it is difficult to determine how aspects of reproduction may be specifically affected. In *M. edulis*, for comparison, the seasonal cycle of storage and utilisation of glycogen reserves is closely linked to annual reproductive cycle and the timing of gametogenesis can impact metabolism and therefore energy allocation (Gabbott, 1976). The reproductive cycle of UK *M. modiolus* lacks seasonal synchronicity with recruitment, spawning period and gonadal maturation varying across seasons, year, depth and location (Holt *et al.*, 1998; Roberts *et al.*, 2011; Dinesen & Morton, 2014). Consequently, gametogenesis in *M. modiolu*s may not be strongly linked to seasonal food availability and seasonal temperature patterns. It should be noted that even where no negative impacts to gametogenesis have been observed,

embryonic development and subsequent quality and survivorship of the larvae may be negatively impacted (Gabbott, 1976). Therefore, results should not be seen as evidence of a "non-impact", and further investigation into knock-on impacts to other measures of reproductive success (e.g. fecundity, sperm motility, hatching success, larval development) is recommended.

Adductor Muscle Tissue

Increased temperature (16-19°C) caused changes in CEA in adductor tissue with an additional influence of food availability. Under 16°C temperature conditions, restricted feed lead to decreased CEA as compared to high feed availability. As adductor muscle may gain the benefit of increased energy from food acquisition and may be used as an energy store for other physiological processes (Barber & Blake, 1981), particularly during periods of starvation (Li *et al.* 2009b), decreases in CEA under restricted feed availability could indicate that *M. modiolus* uses adductor muscle to support energy consumption in other tissues (e.g. gills) or to support physiological processes (e.g. increased metabolism) under warming conditions if food is scarce.

Similarly, at a temperature approaching the thermal maximum (19°C) for *M. modiolus*, a decrease in CEA in adductor muscle occurred despite high feed availability, with values similar to those under low feed conditions. Decreases may again indicate the conversion of adductor energy reserves to support physiological processes associated with *M. modiolus'* stress response. However, information regarding the specifics of the *M. modiolus* thermal stress response is currently largely lacking. Future such investigations would provide valuable information regarding the physiological mechanisms potentially driving the observed changes in *M. modiolus* energy budgets under warming conditions.

<u>Oxygen Consumption</u>

Mean oxygen consumption rates were significantly higher at 13°C and 19°C than at 10°C and 16°C. Oxygen consumption rates in bivalves are largely temperature dependent under increasing temperatures until thermal maxima (Gabbott, 1976). Consequently, the increase in rates from 10°C to 13°C is likely a direct response to increasing temperature.

The observed decrease in mean oxygen consumption rates between 13°C and 16°C could indicate that *M. modiolus* possesses some acclimatory ability as it moves towards upper

thermal limits. Bivalve species have previously been reported to acclimate oxygen consumption rates to temperature change (Gabbott, 1976); *M. edulis*, for example, was observed to alter feeding and respiration rates to maintain energy balance under increased temperature with reduced acclimatory ability occurring when upper thermal limits (>20°C) were reached (Bayne *et al.*, 1976). However, previous research also indicates that while adaptive mechanisms like metabolic rate depression may contribute to survival during short-term heat exposure, they may be less successful during chronic temperature stress (Mattoo *et al.*, 2013).

Decreases in oxygen consumption indicate metabolic depression and may act as an immediate adaptation strategy to survive unfavourable conditions. Metabolic depression of up to 40% of resting metabolic rates have been reported for molluscs when faced with environmental stressors, indicating a major decrease in energy demands of oxygen consumption (Guppy & Withers, 1999). However, when warming extends beyond a species' thermal tolerance window, thermally accelerated rates of oxygen consumption may result, as seen here in *M. modiolus* held at 19°C (Storey & Tanino, 2012). Similarly, Ezgeta-Balić *et al.* (2011) reported increased respiration rates in *Modiolus barbatus* under extreme temperatures conditions. Additionally, warming above critical temperatures causes excessive mitochondrial oxygen demands which can leads to thermal hypoxaemia (i.e. low oxygen levels in haemolymph). Hypoxaemia causes substantial energetic demands for repair of oxidative damage (refer to Chapter 4) and thus, energy allocation towards protective mechanisms (e.g. stress protein synthesis) is dramatically limited (Storey & Tanino, 2012).

Valve closure is another strategy utilised by bivalves to combat short-term stress events (Anestis *et al.*, 2007) and while not quantified during experiments, may further explain the pattern of oxygen consumption rates observed in *M. modiolus* under warming conditions. Valve opening is stimulated by increasing temperature until thermal limits are approached, at which point valve opening declines, accompanied by decreased oxygen consumption and metabolic depression. However, this is a passive approach of thermal tolerance occurring at the expense of reduced aerobic scope and may induce tissue hypoxia. Further, it cannot be maintained in the long-term or if temperatures surpass upper thermal limits (Anestis *et al.*, 2007), at which point valves re-open and oxygen consumption rates dramatically increase.

While results suggest an influence of temperature to oxygen consumption rates, it is emphasized that investigation here provides only a preliminary look at the potential effects of warming to this aspect of energy consumption in *M. modiolus*. Further investigation is needed that includes longer acclimation periods, examines the role of feed ration and compares the response of different populations in order to assess variation in acclimatory ability.

Condition Index

No effects of temperature nor hypoxia to condition index were detected. This is surprising given that previous investigations have reported whole-animal responses to be more sensitive than biochemical reactions (Pörtner, 2010; Schulte *et al*., 2011). Increasing organisational complexity at the whole-organism scale causes narrower thermal windows for whole-level functioning than at the smaller scale of the cell or molecule, and thus whole-organism processes are likely to reach thermal maxima prior to more specific cellular or molecular processes or structures (Pörtner, 2002; Pörtner & Farrell, 2008). This results in systemic limitations at the whole organism level which then lead to further limitations and biochemical stress at tissue, cellular and molecular levels (Pörtner, 2010). Therefore, one would expect the observed impacts of warming to CEA in *M. modiolus* to be paralleled by impacts at the whole-organism level.

While the physiological mechanisms underlying any thermal tolerance of *M. modiolus* are poorly understood, the lack of observable effect of warming to condition index could be partially attributable to the species' life history traits. For example, animal size (in addition to temperature) is an important factor for determining the energy balance of bivalve species (Bayne & Newell, 1983) with larger individuals having proportionally lower energy consumption than smaller cohorts (Bougrier *et al.*, 1995). Additionally, larger mussels take longer to reach internal temperature equilibrium with external environments and thus have increased buffering ability to cope with short-term warming events over smaller individuals (Pianka, 1970; Helmuth, 1998). Compared to other temperate bivalve species, *M. modiolus* is considerably large, achieving shell lengths up to 200mm (Fariñas-Franco *et al.*, 2014). Consequently, *M. modiolus'* size may provide it with some improved physiological advantage for coping with short-term warming events. Further, *M. modiolus* reefs that exist where variable temperatures occur (i.e. shallower coastal areas) may, as an aggregation of individuals, collectively buffer against

warming via maintaining lower levels of thermal inertia across the reef unit (Helmuth, 1998). This is an important point for those individuals/populations that may only experience temporary thermal threat at specified times of the day or year (e.g. low/spring tides) but otherwise live under tolerable conditions.

Another consideration when assessing impacts to condition index is the influence of reproductive status; for example, whether an individual is nearing its spawning window will dictate how energetic resources are allocated between growth and reproduction (Li *et al*., 2009a). *M. modiolus* is generally thought to lack a discrete spawning window and it has been suggested that some populations take a "trickle" approach to release of gametes across the year (Elsäßer *et al*., 2013), in which case one would expect a lessened influence of reproductive aspects (e.g. gonad development) on condition index under warming condition, especially as compared to bivalve species where reproductive status and whole energy status is strongly linked (e.g. *M. edulis*). However, given the considerable variation in reproductive cycles reported for *M. modiolus* populations (Holt *et al.,* 1998; Roberts *et al.,* 2011; Dinesen & Morton, 2014), further investigation that includes analyses of reproductive status (e.g. gonad index and stage of maturity) is recommended to determine the influence to whole organism energy status.

The condition index used in this investigation is a body component index and indicates the proportion of energy directed towards tissue growth vs that allocated to shell growth (Lesser *et al.,* 1994). An increase/decrease in value indicates increased/decreased allocation of energy to tissue growth. Under warming conditions, particularly at upper temperatures towards the thermal maximum for the species, one would therefore have expected values to decrease as tissue energy stores are transferred to maintenance and repair. For example, at the whole-organism level, energetic demands of the stress response may result due to increased rates of ventilation, oxygen uptake and feeding (Willmer *et al*., 2000). Further, specific aspects of the oxidative stress response (refer to Chapter 4) such as the chaperoning action of heat shock proteins, process of ubiquitination (i.e. addition of ubiquitin to proteins, which affects the locations and roles of proteins or flags them for degradation) and synthesis of antioxidants all utilise considerable amounts of energy and thus contribute to both cellular and whole-organism energy demands during exposures to stressors such as warming (Sokolova *et al.* 2012).

Overview of Impacts to Energy Budgets

The investigation has shown that increases in temperature may impact the cellular energetics of *M. modiolus* with food availability playing a significant role, particularly in gill tissue where notable decreases in CEA were observed under high food availability conditions. Changes may also occur in adductor tissue, though to a lesser extent, potentially to support the increased energetic costs of maintenance of other processes under warming conditions. Observed decreases in the gill may be a result of the energetic costs of filter-feeding and consequently, any benefits in increased feed ration are compromised in tissues that play an active role in feeding. Under increasing temperatures to 16°C, changes in CEA in *M. modiolus* were less obvious, but at 19°C, it appears that food availability no longer influences response, and therefore this temperature may limit energy budgets in the species.

Changes in CEA were paralleled by changes in oxygen consumption rates over the same temperature range. Joint examination of both approaches broadly suggests that *M. modiolus* may be able to acclimate to temperatures as high as 16°C but at a temperature approaching thermal maximum for the species (19°C), the species demonstrates increased stress response observed as coinciding reductions in CEA and increased oxygen consumption rates. This response at 19°C may indicate an approximate thermal limit to energy budgets in *M. modiolus*. Additionally, as the strongest impacts to CEA were detected in gill tissue at 19°C, it is of interest that impacts to oxygen consumption (a process primarily associated with the gill) also occurred at this temperature. While no impacts to whole-body condition were observed, it is emphasised that maintenance of somatic growth under warming as demonstrated here may only represent a short-term reprieve that would not be maintained over any long-term exposure (e.g. climate change warming). Further, the impacts of reproductive status and life history traits may influence impacts at the whole-body level and should be investigated in future work.

It is highlighted that acclimatory/evolutionary based differences in energetics have been observed between populations of the same species and are influenced by the environment, activity level and thermal history associated with a particular population (Mattoo *et al.*, 2013). Thus, it would be useful for future work to compare the effects of warming to oxygen consumption rates in *M. modiolus* from different sites to discern any population-level variation in response. Nevertheless, this investigation of the integrated response

under natural temperature and food conditions will contribute towards a better understanding of the physiological mechanisms which influence energy balance in *M. modiolus.* It is also proposed that CEA and oxygen consumption rates are potentially effective physiological markers for demonstrating declines in *M. modiolus* populations under changing environmental conditions, and may have value as an early warning system before greater changes occur at the whole-body level.

6. Summary and Recommendations for Management

6.1 Abstract

To determine the vulnerability of a species or population to climate change, exposure (i.e. environmental conditions), adaptive capacity and acclimatory ability (i.e. sensitivity) must be jointly considered. Following previous chapters' investigations into aspects of each factor, this chapter summarises key findings and provides a preliminary overview of *M. modiolus'* climate change vulnerability at both species and population levels. Schematics are presented alongside discussion to clarify findings and include a simple prototype climate change vulnerability index. For demonstration, vulnerabilities of three *M. modiolus* populations from across the species' latitudinal range (Wales, Scotland West Coast and Orkney) are evaluated. Additional discussion of how findings may be applied to effective management of *M. modiolus* under climate change conditions is also provided. Management consideration includes discussion of how investigations provide key baseline data for climate change impacts and guidance of how genetic connectivity and climate change sensitivity may be incorporated into marine management in Scotland (i.e. via regional management plans), completed as part of an internship project carried out under the supervision of Scottish Natural Heritage.

6.2 Aim and Objectives

Aim:

- Summarise and discuss results (Chapters 2-5) to provide an overall indication of the vulnerability of *M. modiolus* to future climate change;

- Provide management advice for *M. modiolus* under future climate change conditions.

Key Objectives:

- **Provide an overview of key findings** of Chapters 2-5, including summary of key points in table format;

- **Create a visual** that demonstrates how environmental conditions, adaptive capacity and acclimatory ability shape the climate change vulnerability of *M. modiolus*;

- **Create a prototype vulnerability index** for demonstrating vulnerability of *M. modiolus* populations;

- **Consider results as baseline data** for climate change impacts;

- **Consider management measures/recommendations** for *M. modiolus* with regards genetic connectivity and climate change vulnerability; provide guidance for regional marine planning initiatives.

6.3 Overview of Main Findings

Investigations carried out over Chapters 2-5 provide vital evidence regarding the status of *M. modiolus* biogenic reef habitats under current conditions and under the context of climate change. Collective consideration of aspects of *M. modiolus'* environmental conditions (i.e. exposure), genetic structure (i.e. adaptive capacity and connectivity) and physiological limits (i.e. sensitivity/acclimatory ability) will aid in determining vulnerability to climate change at both species- and population-level. Following, a brief summary of findings is provided. Additionally, Table 6.1 is provided as a general overview of key results. Figure 6.1 illustrates how exposure, acclimatory ability and adaptive capacity collectively contribute to overall climate change vulnerability of *M. modiolus* at species and population levels.

Exposure (Chapter 2)

Across the UK, *M. modiolus* annually experiences a wide range of temperatures from lows of 5°C in Shetland during winter months to highs of 17°C in Wales during summer months. Consequently, the species appears to have a wide temperature tolerance range. However, populations across the range experience considerably different temperature ranges across the year. Thus, they have widely varying historical thermal exposures (which could influence acclimatory ability) and face varying degrees of temperature stress (ranging from minimal to extreme). For example, the southern North Lleyn (Wales) site experiences considerably higher (up to +3°C) temperatures across the year as compared to more northern sites, and is subjected to temperatures approaching upper thermal limits during summer and early autumn months. Consequently, it is likely that the Wales population already experiences substantial periods of thermal stress during the year. Similarly, loch populations on the west coast of Scotland may already experience periodic warming events due to decreased water exchange rates in such locations. Populations in these areas may also regularly contend with lowered oxygen conditions brought on by warming or exacerbated by other human activities (e.g. aquaculture). Likewise, historical data indicates that northern Shetland sites also regularly experience summer declines in oxygen content. Under climate change it is highly probable that warming stress will extend in duration and intensity, putting all populations, but especially southern and loch-based populations, at increased risk. Additionally, warming will drive increased threat of decreased oxygen concentrations in all areas, particularly those with historical occurrence of hypoxia due to strengthened thermal stratification under warming conditions.

Adaptive Capacity (Chapter 3)

Given that population connectivity analyses indicated moderate to high levels of gene flow across all *M. modiolus* populations, one would expect that adaptive capacity is generally limited within populations at a site level (Sanford & Kelly, 2009) (i.e. populations have reduced potential to adapt to site-specific stress conditions). For example, more southern or shallower populations may struggle to adapt to warming conditions if they are sinks for larval supply from northern/deeper (i.e. cooler) environments. Reduced adaptive capacity has historically been presumed for bivalve species owing to having planktonic larvae and therefore widespread dispersal. However, differentiation and adaptation have been observed in other bivalve species, and over a range of spatial scales (i.e. meters to kilometres), so should not be ruled out (Kuo & Sanford, 2009). Likewise, increased genetic connectivity may support higher levels of genetic diversity which may contribute to improved resilience by providing a diverse gene pool from which natural selection may occur *(*Sanford & Kelly, 2011). Further investigation into the role of genetics in shaping resilience is recommended, particularly regarding the ability of populations to use genetic mechanisms to mount defences to stressor conditions (e.g. upregulation of HSP70 gene to produce heat shock proteins). Such investigation would illuminate important genetic differences between populations and highlight populations that may be act as critical genetic resources for increased resilience to climate change.

Acclimatory Ability (Chapters 4 and 5)

M. modiolus may be able to tolerate short-term stress events (warming/hypoxia) but under sustained exposures, oxidative damage and shifts in energy budgets may occur as part of a wider stress response. It is uncertain whether *M. modiolus* has the capacity to mount sufficient antioxidant defenses to cope with warming- or hypoxia- induced oxidative stress and results here suggest that ability may vary across populations. Consequently, it emphasised that results from Chapter 4 (oxidative stress response) stand as preliminary investigation rather than conclusive results. Likewise, despite potential ability to adjust energy budgets under short-term exposure to stressors, it is unclear (but unlikely, due to energetic demands) whether such strategies could be sustained in the long-term. Furthermore, the availability of food and local demographics may drive or alter responses and thus need further investigation. Overall, this preliminary comparison of population-level responses suggests varying acclimatory abilities which could potentially be driven by historical exposures, demographics or other factors. Consequently, there may be

populations that are better suited (i.e. more resilient) to increased stress conditions. Likewise, populations may have varying sensitivities to different stressor types (e.g. warming vs hypoxia) as alluded to by the north-south population comparison.

Table 6.1 Main findings of investigations (Chapter 2-5)

Vulnerability = f (exposure, adaptive capacity, acclimatory ability)	
Exposure (Chapter 2)	• Temperature and oxygen conditions vary across sites. • Variability of physical conditions varies across sites. • Populations at the southern extent, in enclosed bays/lochs and in shallower areas are at increased risk of exposure to warming. • Scottish west coast and Shetland Islands populations have seasonal exposure to hypoxia. • Populations in enclosed bays/lochs are at increased risk of exposure to coinciding warming and hypoxia. • The most southern population (North Lleyn) and Dornoch Firth populations are exposed to greatest varation in annual temperature conditions (=increased acclimatory ability/plasticity?). • Populations at the southern extent are at high risk of temperatures approaching thermal limits under climate change conditions.
Genetics (Chapter 3)	• UK *M. modiolus* reef populations have moderate to high levels of genetic connectivity. • Scottish *M. modiolus* beds within MPAs are generally well connected to nearby non-protected beds at both local and regional scales. • Migration rates between populations are generally comparable. • Populations from enclosed water bodies (e.g. sea lochs and firths) appear to act as a source of migrants rather than a sink from adjacent populations. • There are two main genetic groups within the sites sampled. • All *M. modiolus* populations showed a heterozygote deficiency suggesting reduced migration and increased inbreeding (but heterozygote deficiency is also characteristic of bivalve species). • Given the high connectivity of reefs, it is proposed that populations may have reduced capacity to adapt to climate change conditions.
Stress Response (Chapter 4)	• *M. modiolus* reef populations show varying oxidative stress responses and thermal limits so may have varying acclimatory abilities. • *M. modiolus* is likely more sensitive to thermal stress than hypoxia. • Hypoxia reduces thermal limits in *M. modiolus*. • Comparison of populations suggests that the greatest threat to *M. modiolus* under warming conditions will likely be in the southernmost population (North Lleyn) approaching thermal maxima under climate change conditions • *M. modiolus* can cope with acute warming but lengthier exposures cause oxidative damage. • *M. modiolus* has limited ability to use antioxidant defences and ability varies across populations.
Energetics (Chapter 5)	• Warming impacts both CEA and oxygen consumption rates in *M. modiolus* (but no effect to condition index, under short-medium term exposures). • *M. modiolus* may have some ability to adjust oxygen consumption rates under short-term warming conditions (but only to a point). • Impacts are most likely in gill tissue likely due to its active role in food acquisition and oxygen consumption. • Tissue type and food availability are important considerations when assessing the effects of warming on marine bivalves. • Cellular changes in energy budgets and altered oxygen consumption rates may be used as a tool to detect temperature impacts before observable changes in whole body condition occur.

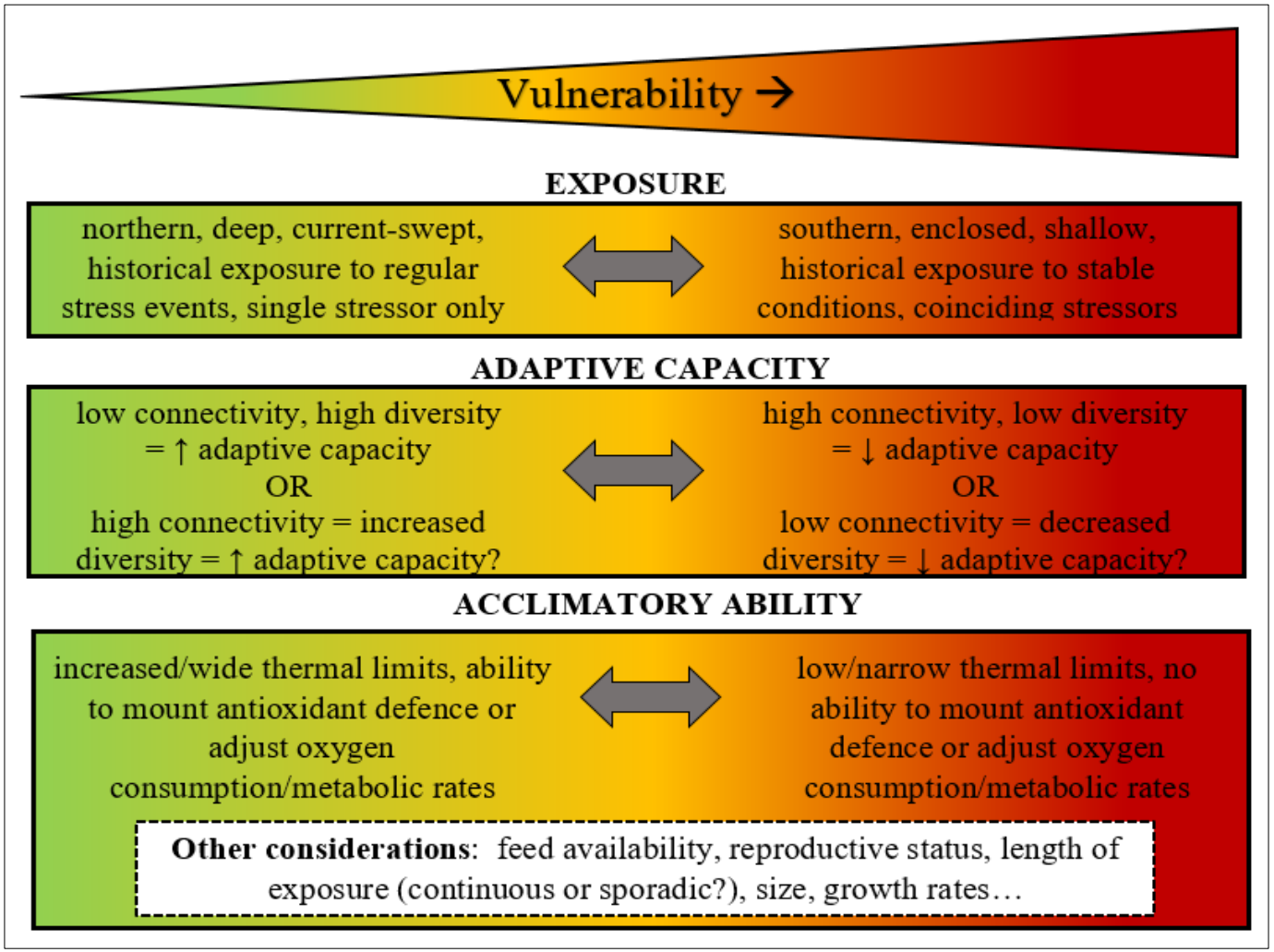


Figure 6.1. Vulnerability *of M. modiolus* to climate change: considering the role of exposure, adaptive capacity and acclimatory ability.

6.4 Assessment of Vulnerability

Evaluation of adaptive capacity, acclimatory ability and exposure (where information is available for all three factors) at the population-level can aid in identifying those populations at greatest risk under climate change (i.e. assessing vulnerability). The use of frameworks such as decision support systems and vulnerability indices allow for the objective interpretation of complex results, and consequently have been shown to be effective tools for advancement of management objectives (Morrison *et al*., 2015; Comer *et al*., 2012). They also promote coordination, consistency and efficiency of planning and managing for adaption and provide opportunity for valuable input on species/populations at risk from a range of research viewpoints (Young *et al.*, 2011). Importantly, they also emphasise the value of collaboration across subject areas that is necessary to gain an accurate collective understanding of a species'/population's sensitivity/resilience.

While the development of frameworks as viable tools for marine managers is a lengthy and involved process (beyond the remit of this thesis), a simple prototype vulnerability index (PVI) may act as a starting point from which the threat of climate change may begin to be assessed. As example, Figure 6.2 outlines a PVI for *M. modiolus* based on the climate change models of Comer *et al*. (2012) and Morrison *et al.* (2015), but solely relevant to the focus of the current project. Specifically, the PVI presented here illustrates how various aspects of exposure, acclimatory ability and adaptive capacity may be collectively considered and assessed to determine *M. modiolus'* vulnerability. Vulnerability is assessed as the correlation between exposure threat and resilience, the latter determined according to adaptive capacity and acclimatory ability (Figure 6.2). Following, for demonstration purposes, Table 6.2 provides an indication of how the PVI model may be applied to determine and compare vulnerabilities of three key *M. modiolus* populations (North Lleyn, Port Appin, and Karlsruhe) that cover the southern, mid and northern extent of the species' UK range.

In future, the PVI framework (or similar) could be developed to suit specific climate scenarios (e.g. changing levels/durations of exposure, additional stressors, cumulative impacts) and include additional exposure/resilience categories as new understanding and knowledge arises (i.e. covering further aspects of *M. modiolus* biology, physiology and ecology). Consequently, with increased research effort, there is strong potential that development of a vulnerability index, or contribution to already existing indices, could

support effective and evidence-based marine management decisions and thereby contribute to the conservation of these ecologically valuable habitats.

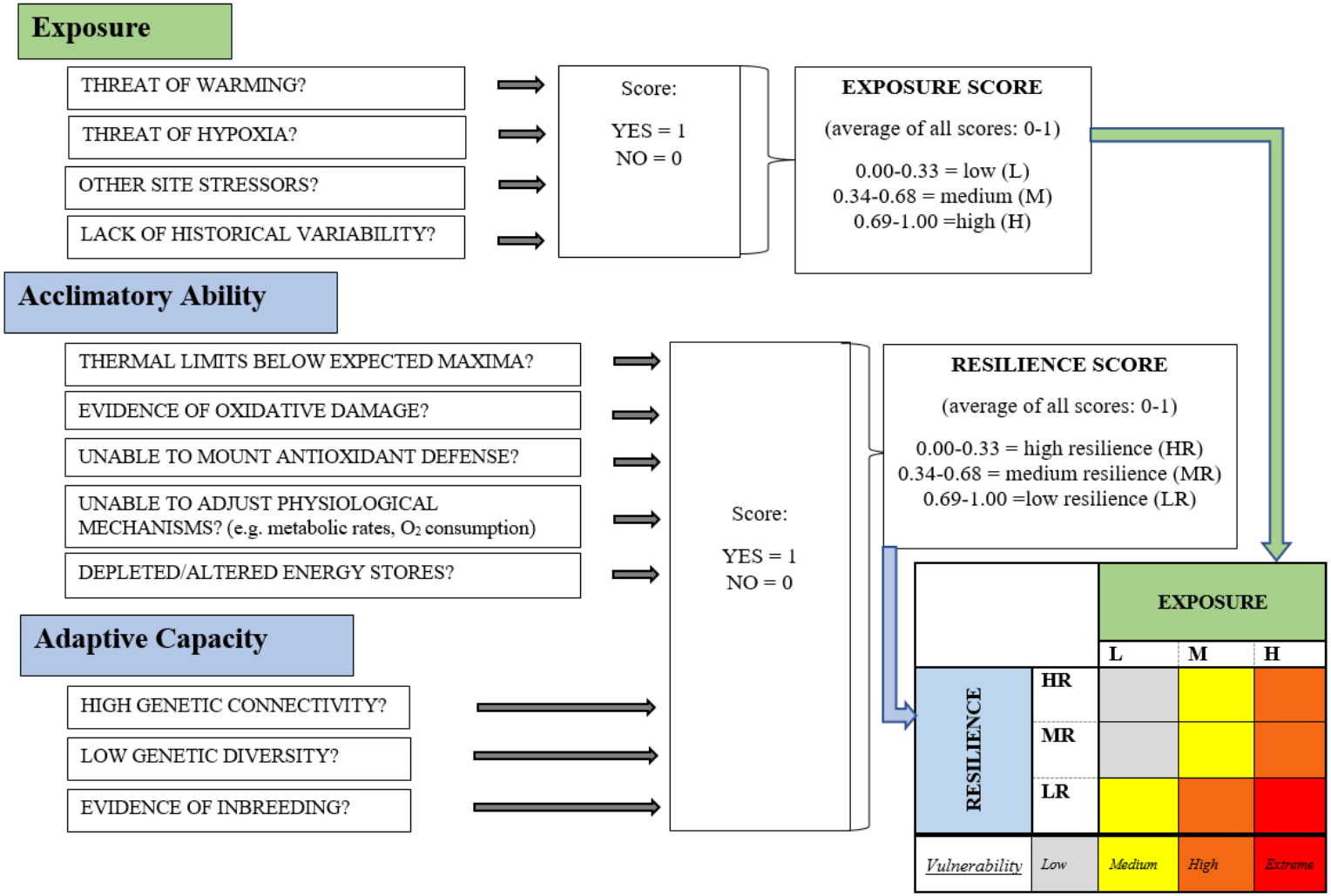


Figure 6.2. Prototype vulnerability scoring system. Exposure and resilience scores are calculated to then determine level of vulnerability (grey = low vulnerability, yellow = medium vulnerability; orange = high vulnerability; red = extreme vulnerability). Schematic adapted from Comer *et al*. (2012).

Table 6.2. Evaluation of vulnerability of *M. modiolus* populations to climate change, according to Figure 6.2 and based on results of Chapters 2-5. "Unknown" indicates that information is not available. *Note: This evaluation only serves as demonstration of application of the PVI rather than conclusive results. Further data and investigation is needed to confirm exposure and resilience for a given population.*

	North Lleyn	*Port Appin*	*Karlsruhe*
Exposure			
Threat of warming?	yes	yes	no
Threat of hypoxia?	yes	yes	no
Other site stressors?	unknown	yes	unknown
Lack of historical variability?	no	yes	unknown
Mean score:	0.50	1.00	0.00
Resilience			
Thermal limits below expected maxima?	yes	no	no
Evidence of oxidative damage?	yes	unknown	yes
Unable to mount antioxidant defense?	yes	unknown	yes
Unable to adjust physiological mechanisms? (O_2 consumption rates)	unknown	yes	unknown
Depleted/altered energy stores?	unknown	yes	unknown
High genetic connectivity?	yes	yes	yes
Low genetic diversity?	yes	yes	yes
Evidence of inbreeding?	yes	yes	yes
Mean score:	0.75	0.50	0.67
Vulnerability	HIGH	HIGH	LOW

6.5 Implications for Management

Provision of Baseline Data

Prior to current investigations, there has been limited research regarding molecular and physiological responses in *M. modiolus* and as as result, model bivalve species (e.g. *Mytilus edulis*) have been relied upon to approximate baseline values. This is problematic given the varying biology (e.g. size, reproductive aspects) and ecology (e.g. habitat, life history) of such species as compared to *M. modiolus*. Consequently, species- and population-level investigations into *M. modiolus* cellular budgets, oxygen consumption, condition index, oxidative stress and genetic connectivity under current sea water temperatures and oxygen levels will provide true baseline data that can accurately inform future monitoring efforts and aid in detecting impacts in *M. modiolus* under climate change conditions. However, it should be noted that where *M. modiolu*s reefs have suffered high levels of degradation (e.g. Strangford Lough) or presently may face considerable levels of physical stress (e.g. southern populations), baseline measures as provided here may not reflect true baseline values (Browman & Stergiou, 2005).

Climate Change Impacts

Under current UK and EU marine directives (e.g. Marine and Coastal Access Act 2009, Marine Strategy Framework Directive), management objectives for priority habitats such as *M. modiolus* biogenic reefs call for them to be assessed, maintained and managed for both current conditions as well as future conditions under climate change scenarios. Consequently, a vital aspect of management decisions is that they include accurate understanding of species/population vulnerabilities and parallel adaptations for climate change. Although current collective scientific knowledge regarding the impacts of climate change (e.g. warming, ocean acidification, hypoxia, etc.) is rapidly increasing, there remain substantial gaps in understanding, particularly regarding the responses of non-commercial species and those species that are less accessible (e.g. sub-tidal) such as *M. modiolus*. Consequently, knowledge arising from the current investigation will contribute to an improved understanding of the species' biology and provides key foundational information about potential climate change impacts that may be taken forward in future research. However, it is important to note that while the EU's MSFD requirement for Good Environmental Status by 2020 may be met, the maintenance of such status may become increasingly unrealistic and unattainable for highly vulnerable *M. modiolus* reefs populations under climate change conditions. Consequently, meeting such requirements may be problematic and a shifting baseline (accounting for climate

change), as outlined by Elliot *et al*. (2015), may need to be accommodated by regular assessments.

Conservation and Restoration

Information regarding specific physiological responses (e.g. thermal limits, stress response) and genetic structure of populations will be instrumental in supporting conservation of *M. modiolus* and is an important contribution to management efforts. Results may inform marine reserve design and strategies to assess resilience to climate change and other anthropogenic impacts (Selkoe *et al*., 2016). For example, range contractions due to warming conditions may call for changes to MPA boundaries (Birchenough *et al.*, 2013). Likewise, knowledge of population-based thermal tolerance limits could aid marine managers in developing management schemes that are suitable for particular populations (e.g. selective closing of certain areas in summer months to fishing activity), as opposed to use of a broadcast approach for the species as whole that may not be appropriate (nor necessary) for all populations. Additionally, where an important genetic source population is under threat from warming, management decisions to protect it from other co-stressors (e.g. fisheries pressures) may be justified. For example, results suggest that the Isle of Man Ramsey Bay (MPA) population is an important genetic sink population that may support other IOM populations. Any impacts to recruitment or dispersal in the Ramsey Bay population due to changing conditions under climate change (e.g. impacts to larvae, changing hydrodynamics, etc.) could have detrimental effects to other populations. Protection of *M. modiolus* reefs populations across the UK's MPA network may also aid in protection of the cumulative genetic diversity that exists across populations which may promote resilience to climate change (Sanford & Kelly, 2004). Furthermore, there is the possibility that the local extinction of heat-sensitive populations may be offset by more heat-tolerant con-specifics (Somero, 2010), and thus must be considered in management plans under future climate change conditions.

Where *M. modiolus* populations have been impacted (e.g. Cook *et al*., 2013) or are in-decline (e.g. Roberts *et al*., 2011), connectivity data may support improved understanding of whether natural recovery might occur on its own (via dispersal from supporting populations) or whether human intervention (e.g. via transplantation) is necessary for recovery (Levin, 2006). Similarly, improved information about the sensitivity and adaptive capacity of reefs may guide the selection of source populations for restoration

efforts, especially under the context of climate change (Sanford & Kelly, 2011). Co-consideration of connectivity and acclimatory ability may also aid in understanding the recovery potential of degraded populations (on a site by site basis) or provide justification for protective measures.

Monitoring

Much of the current UK marine management advice for biogenic reefs focuses on monitoring of physical and ecological properties including examination and quantification of factors such as extent, integrity, vertical relief, mortality and associated fauna/biodiversity (e.g. Joint Nature Conservation Committee's (JNCC) marine monitoring handbook (Davies *et al*., 2001)). It is argued that consideration of physiological stress (at molecular, systemic or whole-organism levels) and genetic connectivity/structure is equally import for determining the status (i.e. health and condition) of reefs and should therefore be included in monitoring programmes for the habitat (La Barre, 2013). For example, genetic markers can act as accurate tools for assessing population size, effective population size, inbreeding and migration which are powerful indicators of population growth or decline (Wan *et al*., 2004).

The use of molecular biomarkers for monitoring *M. modiolus* reefs is promoted here as may allow for early detection of stress response and thus provide sufficient time for appropriate management measures to be carried out before the species reaches the latter stages of decline (as may be the case when monitoring is approached from a purely ecological viewpoint). Likewise, molecular biomarkers may act as appropriate benthic indicators of large-scale or indirect climate change impacts, which tend to be difficult to spatially quantify via visual assessment (e.g. survey) alone (Van Hoey *et al.*, 2010). A molecular approach may also complement intensive physiological investigation for providing evidence of adaptability and/or loss of function (La Barre, 2013).

Additionally, molecular biomarkers offer a practical approach for marine managers in terms of sampling effort and ease. Measurements require only small tissue samples (<1g) that could be collected in the field with minimal training for an in-depth (e.g. including cellular energetic shifts, genomic response, oxidative stress response) examination of in-situ reef condition. However, results here emphasise the importance of selecting appropriate biomarkers and tissue types for monitoring. Gill tissue is proposed as the most suitable tissue for biomonitoring given that it may provide earlier indication of

impacts as compared to adductor or gonad tissue. However, as collection of gill tissue causes the demise of the sample organism, haemolymph samples (which leave the animal intact), may be better suited for populations already in decline. Further investigation into the suitability of biomarkers tested here is needed before sound conclusions regarding appropriateness for climate change monitoring can be made, but preliminary results are promising. It is also acknowledged that the use of such biomarkers may involve considerable personnel, training and material costs that may not be available under existing budgets.

A number of tools have been previously developed to aid in assessment of sensitivities of marine habitats to specific pressures including the SNH and JNCC *Features, Activities, Sensitivities Tool* (FEAST), the Marine Life Information Network (MARLIN) Marine Evidence Sensitivity Assessment (MarESA) and MarLIN Biology and Sensitivity Key Information database, the DEFRA and Natural England sensitivity assessment method (Tillin *et al*., 2010). However, many of these tools currently lack accurate or up-to-date sensitivity information for *M. modiolus* reefs. For example, FEAST information for *M. modiolus* temperature sensitivity is outdated and defined according to the responses of a number of non-bivalve species. Thus, such tools would clearly benefit from the contribution of the current findings.

Climate Envelope Models

Previous habitat modelling (e.g. MAXTENT) of *M. modiolus* distribution under future warming conditions indicates that most suitable habitat for the species will be lost over the next century (Gormley *et al*., 2013). Inclusion of other environmental factors (e.g. hypoxia) may help to refine this model, and likely would show further constraints to the species' distribution under climate change conditions. While MAXTENT modelling is unable to consider larval dispersal or recruitment processes, inclusion of genetic connectivity data may enhance understanding of model predictions and contribute to model accuracy (Gormley *et al*., 2013). Addition of population-based thermal tolerances and stress responses could also aid in addressing two assumptions of standard climate envelope models: firstly, that of "niche conservatism" across space and time (i.e. where predictions based on one location or timepoint are assumed to be relevant to another geographic region and time point); and secondly, that of "conservation of limiting mechanism" (i.e. where mechanisms limiting the distribution of a species in one

geographic region or time point are assumed to be the same limiting mechanisms across all regions/times (Woodin *et al*., 2013).

6.6 Management Guidance (Case study: Scottish Regional MPA Plan)

The Scottish Government's marine directorate, Marine Scotland, is responsible for the integrated management of Scotland's seas. Marine Scotland is currently looking to develop marine protected area (MPA) management plans that cover all MPA sites within marine regions of Scotland, rather than individual site management plans. As principal advisory to Marine Scotland, Scottish Natural Heritage is committed to providing MPA management advice (e.g. conservation objectives, management measures/recommendations for various activities) at both site level and across regions so as to achieve greater efficiencies and influence decision-making. SNH's aim is to produce Regional MPA Management Plans across 11 Scottish marine regions in inshore waters out to 12nm. Each of these regions will ultimately have a Regional Marine Plan (RMP) which will be developed by the region's Marine Planning Partnership (MPP). As part of an internship placement, the author worked with SNH to develop a guidance document (referred to as a template Regional MPA Management Plan, provided on CD-ROM of additional thesis materials) for creation of regional marine plans by MPPs. This document includes guidance on selecting the most suitable features of a marine region for climate change impact and genetic analyses and incorporating the effects of climate change into Regional MPA Management Plans.

6.7 Conclusions

This investigation into the vulnerability of *M. modiolus* to climate change suggests that changing conditions in marine environments pose a serious threat to *M. modiolus* reefs, with warming appearing to be a more immediate concern than hypoxia, though the latter should not be discounted. Greatest threat of climate change is likely faced by populations at the southern extent of the species' range where temperatures look to near or surpass thermal limits, and where coinciding decreases in oxygen will occur as increased temperatures cause decreased oxygen concentrations. Additionally, populations situated in semi-enclosed coastal areas (e.g. loch populations of Scotland's west coast) are likely to also be under threat as such areas have a propensity for warming and hypoxic events (especially where located in proximity to nutrient loads such as salmon farms) due to decreased water exchange rates. In general, the acclimatory ability of *M. modiolus* appears to be limited beyond an initial response. While this investigation shows that *M.*

modiolus may be able to contend with short-term stressor events, it is likely that the species will deteriorate and decline with intensifying stress conditions and acclimation may not be sustainable in the long-term. However, it is emphasised that results only represent a foundational understanding from which much further investigation is needed including consideration of multi-stressor impacts over longer exposure time and examination of additional biomarkers (e.g. gene expression, other antioxidants). Additionally, the role of various ecological factors including food availability and population demographics are highlighted as important considerations for future work. While the high levels of genetic connectivity observed between populations may be beneficial to supporting populations that are isolated or in-decline, the lack of genetic differentiation between populations may also hinder any adaptive capacity of populations to specific site conditions. Consequently, it is important that appropriate conservation management measures be put in place to protect populations from additional stressors and to develop an appropriate monitoring program to ensure early detection of impacts.

References

Abele, D. & Puntarulo, S. (2004) Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 138(4), 405–15.

ABPmer (2008) Atlas of UK Marine Renewable Resources. ABPmer. http://www.renewables-atlas.info/. Accessed January 2017.

Absolom, D.R. (1986) Basic methods for the study of phagocytosis. *Methods in Enzymology*, 132, 95–182.

Adams, T.P., Aleynik, D., & Burrows, M. T. (2014) Larval dispersal of intertidal organisms and the influence of coastline geography. *Ecography*, 37(7), 698-710.

Addison, J.A. & Hart, M.W. (2005) Spawning, copulation and inbreeding coefficients in marine invertebrates. *Biology Letters*, 1, 450–453.

Ahmad, S. (1995) Oxidative stress from environmental pollutants. *Archives of Insect Biochemistry and Physiology*, 29(2), 135-157.

Ahn, I.Y., Surh, J., Park, Y.G., Kwon, H., Choi, K.S. *et al*. (2003) Growth and seasonal energetics of the Antarctic bivalve *Laternula elliptica* from King George Island, Antarctica. *Marine Ecological Progress Series*, 257, 99-110.

An, M.I. & Choi, C.Y. (2010) Activity of antioxidant enzymes and physiological responses in ark shell, *Scapharca broughtonii*, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular biology*, 155(1), 34–42.

Anestis, A., Pörtner, H.O., Lazou, A. & Michaelidis, B. (2008) Metabolic and molecular stress responses of sublittoral bearded horse mussel *Modiolus barbatus* to

warming sea water: implications for vertical zonation. *The Journal of Experimental Biology*, 211(17), 2889–98.

Anwar, N.A., Richardson, C.A. & Seed, R. (1990) Age determination, growth rate and population structure of the horse mussel *Modiolus modiolus*. *Journal of the Marine Biological Association UK*, 70, 441-457.

Bakhmet, I. N., Komendantov, A. J. & Smurov, A. O. (2011) Effect of salinity change on cardiac activity in *Hiatella arctica* and *Modiolus modiolus*, in the White Sea. *Polar Biology*, 35(1), 143–148.

Barber, B.J. & Blake, N.J. (1981) Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (Say). Journal of Experimental Marine Biology and Ecology 52(2-3), 121–134.

Barnett, T.P., Pierce, D.W. & Schnur, R. (2001) Detection of anthropogenic climate change in the world's oceans. *Science*, 13, 270-274.

Bayne, B.L., Thompson, R.J. & Widdows, J. (1976) Physiology: I. In: Bayne, B.L.(ed) *Marine Mussels: Their Ecology and Physiology.* Cambridge University Press, Cambridge, pp. 121-206.

Bayne, B.L. & Newell, R.C. (1983) Physiological energetics of marine molluscs. In: Saleuddin, A.S.M., Wilbur, K.M. (eds) *The Mollusca, Vol. 4. Physiology. Part 1.* Academic Press, New York, pp. 407–515.

Beerli, P. & Palczewski, M. (2010) Unified Framework to Evaluate Panmixia and Migration Direction Among Multiple Sampling Locations. *Genetics*, 185, 313-326.

Beniash, E., Ivanina, A., Lieb, N., Kurochkin, I. & Sokolova, I. (2010) Elevated level of carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica* (Gmelin). *Marine Ecology Progress Series*, 419, 95–108.

Bierne, N., Launey, S. & Naciri-graven, Y. (1998) Early effect of inbreeding as revealed by microsatellite analyses *on Ostrea edulis* larvae. *Genetics*, 148, 1893–1906.

Bindoff, N.L.& Hobbs, W.R. (2013) Deep ocean freshening. *Nature Climate Change*, 3, 864-865.

Bindoff, N.L., Willebrand, J., Artale, V., Cazenave, A., Gregory, J. *et al.* (2007) Observations: Oceanic Climate Change and Sea Level. In: *Climate Change 2007: The Physical Science Basis*. *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Birchenough, S.N.R., Bremner, J., Henderson, P., Hinz, H., Jenkins, S. *et al.* (2013) Impacts of climate change on shallow and shelf subtidal habitats. *MCCIP Science Review 2013,* 193-203.

Birchenough, S.N., Reiss, H., Degraer, S., Mieszkowska, N., Borja, Á., *et al.* (2015) Climate change and marine benthos: a review of existing research and future directions in the North Atlantic. *Wiley Interdisciplinary Reviews: Climate Change, 6(2), 203-22*3.

Boni, R., Gallo, A., Montanino, M., Macina, A. & Tosti, E. (2016) Dynamic changes in the sperm quality of *Mytilus galloprovincialis* under continuous thermal stress. *Molecular Reproduction and Development*, 83, 162–173.

Bopp, L., Resplandy, L., Orr, J.C., Doney, S.C., Dunne, J.P. *et al.* (2013) Multiple stressors of ocean ecosystems in the 21st century: projections with CMIP5 models. *Biogeosciences*, 10, 6225-6245.

Bougrier, S., Geairon, P., Jonquikres, G. & Bather, C. (1995) Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). *Aquaculture*, 134, 143–154.

Boyer, T., Levitus, S., Antonov, J., Locarnini, R., Mishonov, A. *et al.* (2007) Changes in freshwater content in the North Atlantic Ocean 1955-2006. *Geophysical Research Letters*, 34(16), L16603.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.

Brash, J.M., Cook, R.L., Mackenzie, C.L. & Sanderson, W.G. (2017) Successful management of protected biodiversity hotspots: Considering the demographics and morphology of biogenic beds. *Journal of the Marine Biological Association of the UK* (in press).

Brown, R.A. (1984) Geographical variations in the reproduction of the horse mussel, *Modiolus modiolus* (Mollusca: Bivalvia). *Journal of the Marine Biological Association of the UK*, 64(04), 751-770.

Browman, H. I. & Stergiou, K. I. (2005) Politics and socio-economics of ecosystem-based management of marine resources. *Marine Ecology Progress Series*, 300, 241–296.

Buschini, A., Carboni, P., Martino, A., Poli, P. & Rossi, C. (2003) Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of *Dreissena polymorpha*. *Mutation Research*, 537(1), 81–92.

Cadenas, E. (1995) Mechanisms of oxygen activation and reactive oxygen species detoxification. In: *Oxidative stress and antioxidant defenses in biology*. Springer US. pp. 1-61.

Caldeira, K. & Wickett, M.E. (2003) Anthropogenic carbon and ocean pH. *Nature*, 425, 365.

Callaway, R., Shinn, A. P., Grenfell, S. E., Bron, J. E., Burnell, G., *et al*. (2012) Review of climate change impacts on marine aquaculture in the UK and Ireland. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 22(3), 389–421.

Calosi, P., Rastrick, S. P., Lombardi, C., de Guzman, H. J., Davidson, L. *et al*. (2013) Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO2 vent system. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1627), 20120444.

Calosi, P., Melatunan, S., Turner, L.M., Artioli, Y., Davidson, R.L. *et al.* (2017) Regional adaptation defines sensitivity to future ocean acidification. *Nature Communications*, *8*(May 2016), 1–10.

Calosi, P., De Wit, P. De, Thor, P. & Dupont, S. (2016) Will Life find a Way? Evolution of Marine Species Under Global Change. *Evolutionary Applications*, (September), 1035–1042.

Camejo, G., Wallin, B., Enojärvi, M. (1998) Analysis of oxidation and antioxidants using microtiter plates. In: Armstrong, D. (Ed.), *Free Radical and Antioxidant Protocols. Methods in Molecular Biology, vol. 108*. Humana Press Inc., Totawa, NJ, pp. 377–386

Chahine, M.T. (1992) The hydrological cycle and its influence on climate. *Nature*, 359(6394), 373-380.

Chapuis, M.-P. & Estoup, A. (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24(3), 621-631.

Cherkasov, A.S., Biswas, P.K., Ridings, D.M., Ringwood, A.H. & Sokolova, I.M. (2006) Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. *Journal of Experimental Biology*, 209(7), 1274–84.

Cheung, W.W.L., Pinnegar, J., Merino, G., Jones, M. C. & Barange, M. (2012) Review of climate change impacts on marine fisheries in the UK and Ireland. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 22(3), 368–388.

Clark, D., Lamare, M. & Barker, M. (2009) Response of sea urchin pluteus larvae (Echinodermate: Echinoidea) to reduced seawater pH: a comparison among a tropical, temperate, and a polar species. *Marine Biology*, 156, 1125-1137.

Clausen, I. & Riisgard, H.U. (1996) Growth, filtration and respiration in the mussel *Mytilus edulis*: no evidence for physiological regulation of the filter-pump to nutritional needs. *Marine Ecology Progress Series*, 141, 37–45.

Cocco, V., Joos, F., Steinacker, M., Frölicher, T.L., Bopp, L., *et al.* (2013) Oxygen and indicators of stress for marine life in multi-model global warming projections. *Biogeosciences*, 10, 1849-1868.

Collins, A.R. (2004) The Comet assay for DNA damage and repair principles, applications, and limitations. *Molecular Biotechnology*, 26, 249–260.

Comely, C.A. (1978) *Modiolus modiolus* (L.) from the Scottish West coast. I. Biology. *Ophelia*, 17, 167-193.

Comer, P. J., Young, B., Schulz, K., Kittel, G., Unnasch, B. *et al.* (2012) Climate Change Vulnerability and Adaptation Strategies for Natural Communities: Piloting methods in the Mojave and Sonoran deserts. Report to the US Fish and Wildlife Service. NatureServe, Arlington, VA.

Cook, R., Fariñas-Franco, J.M., Gell, F.R., Holt, R.H.F., Holt, T., *et al*. (2013) The substantial first impact of bottom fishing on rare biodiversity hotspots: a dilemma for evidence-based conservation. *PLoS ONE*, 8(8), e69904.

Cook, T., Folli, M., Klinck, J., Ford, S. & Miller, J. (1998) The relationship between increasing sea surface temperature and the northward spread of *Perkinsus marinus* (Dermo) disease epizootics in oysters. *Estuarine Coastal Shelf Science*, 40, 587–597.

Cornette, J.L., Lieberman, B.S & Goldstein, R.H. (2002) Documenting a significant relationship between macroevolutionary origination rates and Phanerozoic pCO2 levels. *Proceedings of the National Academy of Sciences*, 99, 7832–7835.

Coscia, I., Robins, P.E., Porter, J.S., Malham, S.K., & Ironside, J.E. (2012) Modelled larval dispersal and measured gene flow: seascape genetics of the common cockle *Cerastoderma edule* in the southern Irish Sea. *Conservation Genetics*, 14(2), 451–466.

Costantini, D. (2010) Redox physiology in animal function: The struggle of living in an oxidant environment. *Current Zoology*, 56(6), 687-702.

Coughlan, B.M., Hartl, M.G., O'Reilly, S.J., Sheehan, D., Morthersill, C. *et al.* (2002) Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments. *Marine Pollution Bulletin*, 44(12), 1359–65.

Council Directive 92/43/EEC (1992) Conservation of natural habitats and of wild flora and fauna. *International Journal of the European Communities,* L206, 7–49.

David, E., Tanguy, A., Pichavant, K. & Moraga, D. (2005) Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *The Federation of European Biochemical Societies Journal,* 272(21), 5635–52.

Davies, A.J., Last, K.S., Attard, K. & Hendrick, V.J. (2009) Maintaining turbidity and current flow in laboratory aquarium studies, a case study using *Sabellaria spinulosa*. *Journal of Experimental Marine Biology and Ecology*, 370(1), 35-40.

Davies, J., Baxter, J., Bradley, M., Connor, D., Khan, J. *et al.* (2001) Marine Monitoring Handbook, 405 pp, Joint Nature Conservation Committee, ISBN 1 85716 550 0 JNCC.

Davies, K.J.A. (1987) Protein damage and degradation by oxygen radicals. *Journal of Biological Chemistry*, 262, 9895–9901.

Davies, K.J.A. (1995) Oxidative stress: the paradox of aerobic life. In *Biochemical Society Symposia* (Vol. 61). Portland Press Ltd, London. pp. 1-32.

De Coen, W.M. & Janssen, C.R. (1997) The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. *Journal of Aquatic Ecosystem Stress and Recovery*, 6, 43–55.

Dhawan, A., Bajpayee, M. & Parmar, D. (2009) Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell biology and Toxicology*, *25*(1), 5-32.

Dieringer, D. & Schlötterer, C. (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, 3(1), 167-169.

Dinesen, G. E. & Morton, B. (2014) Review of the functional morphology, biology and perturbation impacts on the boreal, habitat-forming horse mussel *Modiolus modiolus* (Bivalvia: Mytilidae: Modiolinae). *Marine Biology Research*, 10(9), 845–870.

Dondero, F., Dagnino, A., Jonsson, H., Caprì, F., Gastaldi, L. *et al.* (2006) Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. *Aquatic Toxicology (Amsterdam, Netherlands)*, 78(1), S13–24.

Doney, S.C. (2006) The dangers of ocean acidification. *Scientific American*, 294, 58-65.

Doney, S.C., Fabry, V.J., Feely, R.A. & Kleypas, J.A. (2009) Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science*, 1, 169-192.

Durant, J.M., Hjermann, D.Ø., Ottersen, G. & Stenseth, N.C. (2007) Climate and the match or mismatch between predator requirements and resource availability. *Climate Research*, 33, 271–283.

Earl, D.A. & von Holdt, B.M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4(2), 359-361.

Elliott, M., Borja, A., McQuatters-Gollop, A., Mazik, K., Birchenough, S., *et al*. (2015) Force majeure: Will climate change affect our ability to attain Good Environmental Status for marine biodiversity? *Marine Pollution Bulletin*, *95*(1), 7-27.

Ellis, R. P., Parry, H., Spicer, J. I., Hutchinson, T. H., Pipe, R. K. *et al.* (2011) Immunological function in marine invertebrates: Responses to environmental perturbation. *Fish & Shellfish Immunology*, 30(6), 1209–22.

Elsäßer, B., Fariñas-Franco, J.M., Wilson, C.D., Kregting, L. & Roberts, D. (2013) Identifying optimal sites for natural recovery and restoration of impacted biogenic habitats in a special area of conservation using hydrodynamic and habitat suitability modelling. *Journal of Sea Research*, 77, 11–21.

Erk, M., Ivanković, D. & Strižak, Ž. (2011) Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker. *Marine Pollution Bulletin*, 62(5), 1124–9.

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14(8), 2611-2620.

Evans, G.L., le B. Williams, P.J. & Mitchelson-Jacob, E.G. (2003) Physical and anthropogenic effects on observed long-term nutrient changes in the Irish Sea. *Estuarine, Coastal and Shelf Science*, 57(5-6), 1159–1168.

Excoffier, L. & Lischer, H.E.L. (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10, 564-567.

Ezgeta-Balić, D., Rinaldi, A., Peharda, M., Prusina, I., Montalto, V. *et al.* (2011) An energy budget for the subtidal bivalve *Modiolus barbatus* (Mollusca) at different temperatures. *Marine Environmental Research*, 71(1), 79–85.

Fabry, V.J., Seibel, B.A., Feely, R.A. & Orr, J.C. (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science*, 65, 414-432.

Falush, D., Stephens, M. & Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, 164(4), 1567-1587.

Falush, D., Stephens, M. & Pritchard, J.K. (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes*, 7(4), 574-578.

Fariñas-Franco, J.M., Sanderson, W.G. & Roberts, D. (2014) Phenotypic differences may limit the potential for habitat restoration involving species translocation: A case study of shape ecophenotypes in different populations of *Modiolus modiolus* (Mollusca: Bivalvia). *Aquatic Conservation: Marine and Freshwater Ecosystems*, 26(1), 76-94.

Feder, M. E., & Hofmann, G. E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61, 243–82.

Fernández-Reiriz, M.J., Labarta, U. & Babarro, J. M. (1996) Comparative allometries in growth and chemical composition of mussel (*Mytilus galloprovincialis* Lmk) cultured in two zones in the Ria sada (Galicia, NW Spain). *Journal of Shellfish Research*, 15, 349-353.

Ford, S.E. (1996) Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? *Journal of Shellfish Research*, 15, 45–56.

Frederich, M. & Pörtner, H.O. (2000) Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 279(5), R1531-8.

Frenzilli, G., Nigro, M., Scarcelli, V., Gorbi, S. & Regoli, F. (2001) DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel, *Mytilus galloprovincialis*: a field study in a highly eutrophicated coastal lagoon. Aquatic Toxicology, 53(1), 19–32.

Füssel, H.-M. & Klein, R.J.T. (2006) Climate Change Vulnerability Assessments: An Evolution of Conceptual Thinking. *Climatic Change*, 75(3), 301–329.

Gabbott, P.A. (1976) Energy metabolism. In: Bayne BL (ed) *Marine Mussels: Their Ecology and Physiology*. Cambridge University Press, Cambridge, pp 293-355.

Gabbott, P.A. & Bayne, B.L. (1973) Biochemical Effects of Temperature and Nutritive Stress on *Mytilus edulis* L. Journal of the Marine Biological Association of the UK, 53, 269–286.

Gallego, A., Gibb, F.M., Tulett, D. & Wright, P.J. (2013) Connectivity of benthic priority marine species within the Scottish MPA network. *Marine Scotland Science, Scottish Marine and Freshwater Science*, 4(2), 1-51.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A. *et al*. (2013) Impacts of ocean acidification on marine shelled molluscs. *Marine Biology*, 160(8), 2207–2245.

Ghezzi, P., & Bonetto, V. (2003) Redox proteomics: identification of oxidatively modified proteins. *Proteomics*, 3(7), 1145-1153.

Gnaiger, E. (1983) Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In: Gnaiger E, Forstner H (eds) *Polarographic Oxygen Sensors*. Springer, Berlin, Heidelberg, pp 337-345.

Gormley, K.S.G. (2014) Mapping priority marine habitats: knowledge of their ecosystem to underpin the marine planning process. Dissertation, Heriot-Watt University, Edinburgh.

Gormley, K.S.G., Porter, J.S., Bell, M.C., Hull, A.D. & Sanderson W.G. (2013) Predictive Habitat Modelling as a Tool to Assess the Change in Distribution and Extent of an OSPAR Priority Habitat under an Increased Ocean Temperature Scenario: Consequences for Marine Protected Area Networks and Management. *PloS One*, 8(7), e68263.

Gormley, K.S.G., Mackenzie, C.L., Robins, P., Coscia, I., Cassidy, A. *et al.* (2015) Connectivity and dispersal patterns of protected biogenic beds: implications for the conservation of *Modiolus modiolus* (L.) in the Irish Sea. *PLoS ONE*, 10(12), e0143337.

Goudet, J. (1995) FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity*, 86, 485-486.

Gray, J.S., Wu, R.S. & Or, Y.Y. (2002) Effects of hypoxia and organic enrichment on the coastal marine environment. *Marine Ecological Progress Series*, 238, 249–279.

GESAMP (Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). (2001) Planning and management for sustainable coastal aquaculture development. GESAMP Reports and Studies No. 68. Rome: Food and Agriculture Organization of the United Nations.

Guderley, H. & St-Pierre, J. (2002) Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change, *Journal of Experimental Biology*, 205(15), 2237–2249.

Guinotte, J.M. & Fabry, V.J. (2008) Ocean acidification and its potential effects on marine ecosystems. *New York Academy of Sciences*, 1134, 320-342

Guppy, M. & Withers, P. (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews*, 74, 1–40.

Gutierrez, J.L., Jones, C.G., Strayer, D.L. & Iribane, O.O. (2003) Molluscs as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos*, 101, 79–90.

Halanych, K.M., Vodoti, E.T., Sundberg, P. & Dahlgren, T.G. (2013) Phylogeography of the horse mussel *Modiolus modiolus*. *Journal of the Marine Biological Associaiton of the UK*, 93(07), 1857-1869.

Harley, C.D.G., Randall Hughes, A., Hultgren, K.M., Miner, B.G., Sorte, C.J.B. *et al.* (2006) The impacts of climate change in coastal marine systems. *Ecology Letters*, 9(2), 228–41.

Hartl D.L. & Clark A.G. (1997) Principles of population genetics. 3rd edition. Sinauer Associates: Sunderland, MA.

Hartl, M.G.J., Grigson, S.J.W. & Sinet, E. (2010) Brief Communication Maintenance of Bivalve Hemocytes for the Purpose of Delayed DNA Strand Break Assessment Using the Comet Assay. *Environmental and Molecular Mutagenesis*, 51(1), 64–68.

Heath, M.R., Neat, F.C., Pinnegar, J.K., Reid, D.G., Sims, D.W. *et al.* (2012) Review of climate change impacts on marine fish and shellfish around the UK and Ireland. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 22(3), 337–367.

Heise, K., Puntarulo, S., Nikinmaa, M., Abele, D. & Pörtner, H.-O. (2006) Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus* L. *The Journal of Experimental Biology*, 209(2), 353–63.

Hellberg, M.E., Burton, R.S., Hedgecock, D., Neigel, J.E. & Palumbi, S.R. (2002) Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science*, 70, 273–290.

Helm, K.P., Bindoff, N.L. & Church, J.A. (2010) Changes in the global hydrological-cycle inferred from ocean salinity. *Geophysical Research Letters*, 37(18), L18701.

Helmuth, B.S.T. (1998) Intertidal mussel microclimates: predicting the body temperature of a sessile invertebrate. *Ecological Monographs*, 68, 51–74.

Helmuth, B., Kingsolver, J.G. & Carrington, E. (2005) Biophysics, physiological ecology, and climate change: does mechanism matter? *Annual Review of Physiology*, 67, 177–201.

Hennige, S.J., Wicks, L.C., Kamenos, N.A., Perna, G., Findlay, H.S. *et al.* (2015) Hidden impacts of ocean acidification to live and dead coral framework. *Proceedings of the Royal Society B: Biological Sciencies*, 282(1813), 20150990.

Hirst, N.E., Clark, L. & Sanderson, W.G. (2012) The distribution of selected MPA search features and Priority Marine Features off the NE coast of Scotland. Scottish Natural Heritage Commissioned Report No. 500.

Hirst, N.E., Kamphausen, L.M., Cook, R.L., Porter, J.S. & Sanderson, W.G. (2013) The distribution and status of proposed protected features within the Fetlar and Haroldswick MPA proposal. Scottish Natural Heritage Commissioned Report No. 599.

Hiscock, K., Southward, A., Tittley, I., Jory, A. & Hawkins S. (2001) The impact of climate change on subtidal and intertidal benthic species in Scotland. Scottish Natural Heritage Research Survey and Monitoring Report (January).

Hiscock, K., Southward, A., Tittley, I. & Hawkins, S. (2004) Effects of changing temperature on benthic marine life in Britain and Ireland. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 14(4), 333-362.

Hofmann, G.E. & Somero, G.N. (1996) Inter- specific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. *Marine Biology*, 126, 65–75.

Hofmann, G.E. & Todgham, A.E. (2010) Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology*, 72, 127–45.

Hohenlohe, P.A. (2004) Limits to gene flow in marine animals with planktonic larvae: models of Littorina species around Point Conception, California. *Biological Journal of the Linnean Society*, 82(2), 169-187.

Holleley, C.E. & Geerts, P.G. (2009) Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques*, 46(7), 511-517.

Holliday, N.P., Hughes, S.L., Bacon, S., Beszczynska-Möller, A., Hansen, B. *et al.* (2008) Reversal of the 1960s to 1990s freshening trend in the northeast North Atlantic and Nordic Seas. *Geophysical Research Letters*, *35*(3), L03614.

Holt, T.J., Rees, E.I., Hawkins, S.J. & Seed, R. (1998) Biogenic Reefs (volume IX). An overview of dynamic and sensitivity characteristics for conservation management of marine SACs. Scottish Association for Marine Science (UK Marine SACs Project). 170 pp.

Honkoop, P.J.C. & Beukema, J.J. (1997) Loss of body mass in winter in three intertidal bivalve species: an experimental and observational study of the interacting effects between water temperature, feeding time and feeding behaviour. *Journal of Experimental Biology and Ecology*, 212, 277–297.

Hoshino, A.A., Bravo, J P., Nobile, P.M. & Morelli, K.A. (2012) Microsatellites as Tools for Genetic Diversity Analysis. In *Genetic Diversity in Microorganisms*. INTECH Open Access Publisher.

Houghton, J.T., Ding, Y., Griggs, D.J., Nogouer, M., van der Linden, P.J. *et al.* (2001) *Climate Change 2001: The Scientific Basis*. *Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge University Press, Cambridge UK. 83 pp.

Hummel, H., Bogaards, R.H., Bachelet, G., Caron, F., Sola, J.C. *et al.* (2000) The respiratory performance and survival of the bivalve *Macoma balthica* (L.) at the southern limit of its distribution area: a translocation experiment. *Journal of Experimental Marine Biology and Ecology*, 251(1), 85-102.

IPCC (2013) Summary for Policymakers. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jiale, L., Wang, G. & Bai, Z. (2009) Genetic variability in four wild and two farmed stocks of the Chinese freshwater pearl mussel (*Hyriopsis cumingii*) estimated by microsatellite DNA markers. *Aquaculture*, 287, 286–91

Katsikatsou, M., Anestis, A., Pörtner, H., Vratsistas, A., Aligizaki, K. *et al.* (2012) Field studies and projections of climate change effects on the bearded horse mussel *Modiolus barbatus* in the Gulf of Thermaikos, Greece. *Marine Ecology Progress Series*, 449, 183–196.

Keeling, R.F. & Garcia, H.E. (2002) The change in oceanic O_2 inventory associated with recent global warming. *Proceedings of the National Academy of Sciences,* 99, 7848-7853.

Keeling, R.F., Körtzinger, A. & Gruber, N. (2010) Ocean deoxygenation in a warming world. *Annual Review of Marine Science*, 2(1), 199–229.

Kelly, M.W. & Hofmann, G.E. (2012) Adaptation and the physiology of ocean acidification. *Functional Ecology* 27(4), 980-990.

Kent, F.E.A. (2015) Ecosystem services provided by *Modiolus modiolus* biogenic reefs. Dissertation, Heriot-Watt University, Edinburgh.

Kültz, D. (2005) Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology*, 67(1), 225–57.

Kuo, E. S. & Sanford, E. (2009) Geographic variation in the upper thermal limits of an intertidal snail: implications for climate envelope models. *Marine Ecology Progress Series*, *388*, 137-146.

Kurihara, H. 2008. Effects of CO2-driven ocean acidification on the early developmental stages of invertebrates. *Marine Ecology Progress Series*, 373, 275–284.

La Barre, S. (2013) Novel Tools for the Evaluation of the Health Status of Coral Reefs Ecosystems and for the Prediction of Their Biodiversity in the Face of Climatic Changes. *Topics in Oceanography*, 127-155.

Lancaster, J. (Ed.), McCallum, S., Lowe, A.C., Taylor, E., Chapman, A. *et al.* (2014) Development of detailed ecological guidance to support the application of the Scottish MPA selection guidelines in Scotland's seas. Scottish Natural Heritage Commissioned Report No.491. Horse Mussel Beds–supplementary document.

Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M. & Bock, C. (2010) Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*--changes in metabolic pathways and thermal response. Marine Drugs, 8(8), 2318–39.

Lawrence, J.M. (1976) Patterns of lipid storage in post-metamorphic marine invertebrates. *American Zoologist*, 16(4), 747-762.

Lee, W.S., Monaghan, P. & Metcalfe, N.B. (2013) Experimental demonstration of the growth rate–lifespan trade-off. *Proceedings of the Royal Society B - Biological Sciences*, 280(1752), 20122370.

Lesser, M.P. (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. *Annual Review of Physiology*, 68(3), 253–78.

Lesser, M.P. & Kruse, V. (2004) Seasonal temperature compensation in the horse mussel, *Modiolus modiolus*: metabolic enzymes, oxidative stress and heat shock proteins. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 137(3), 495–504.

Lesser, M.P., Witman, J.D. & Sebens, K.P. (1994) Effects of flow and seston availability on scope for growth of benthic suspension-feeding invertebrates from the Gulf of Maine. *Biological Bulletin*, 187(3), 319–335.

Levin, L.A. (2006) Recent progress in understanding larval dispersal: new directions and digressions. *Integrative and Comparative Biology*, 46(3), 282–297.

Levin, L.A., Ekau, W., Gooday, A.J., Jorissen, F., Middelburg, J.J. *et al.* (2009) Effects of natural and human-induced hypoxia on coastal benthos. *Biogeosciences*, 6, 2063–2098.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I. *et al.* (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, 186, 464–478

Li, Y., Qin, J.G., Li, X. & Benkendorff, K. (2009a) Monthly variation of condition index, energy reserves and antibacterial activity in Pacific oysters, *Crassostrea gigas*, in Stansbury (South Australia). *Aquaculture*, 286(1-2), 64–71.

Li, Y., Qin, J.G., Li, X. & Benkendorff, K. (2009b) Spawning-dependent stress response to food deprivation in Pacific oyster *Crassostrea gigas*. *Aquaculture*, 286(3), 309-317.

Lindenbaum, C., Bennell, J.D., Rees, E.I.S., McClean, D., Cook, W., *et al.* (2008) Small-scale variation within a *Modiolus modiolus* (Mollusca: Bivalvia) reef in the Irish Sea: I. Seabed mapping and reef morphology. *Journal of the Marine Biological Association of the UK*, 88, 133-141.

Liu, W., Huang, X., Lin, J. & He, M. (2012) Seawater Acidification and Elevated Temperature Affect Gene Expression Patterns of the Pearl Oyster *Pinctada fucata. PloS one*, 7(3), e33679.

Logan, C.A., Kost, L.E. & Somero, G.N. (2012) Latitudinal differences in *Mytilus californianus* thermal physiology. *Marine Ecology Progress Series*, 450, 93-105.

Lowe, W.H. & Allendorf, F.W. (2010) What can genetics tell us about population connectivity? *Molecular Ecology*, 19, 3038– 3051

Lowe, A., Harris, S. & Ashton, P. (2004) *Ecological genetics: design, analysis, and application*. Blackwell Science Ltd, Oxford, UK. 326 pp.

Lushchak, V.I. (2011) Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology* (Amsterdam, Netherlands), 101(1), 13–30.

Luttikhuizen, P.C., Drent, J., van Delden, W. & Piersma, T. (2003) Spatially structured genetic variation in a broadcast spawning bivalve: quantitative vs. molecular traits. *Journal of Evolutionary Biology*, 16(2), 260–72.

Mackenzie, C.L., Bell, M. C., Birchenough, S.N.R., Culloty, S. C., Sanderson, W. G. *et al*. (2013) Future socio-economic and environmental sustainability of the Irish Sea requires a multi-disciplinary approach with industry and research collaboration, and cross-border partnership. *Ocean & Coastal Management, 85*, 1–6.

Mackenzie, C.L., Ormondroyd, G.A., Curling, S.F., Ball, R.J., Whiteley, N.M. *et al.* (2014) Ocean Warming, More than Acidification, Reduces Shell Strength in a Commercial Shellfish Species during Food Limitation. *PloS One,* 9(1): e86764.

Mair, J.M., Moore, C.G., Kingston, P.F. & Harries, D.B. (2000) A review of the status, ecology and conservation of horse mussel *Modiolus modiolus* beds in Scotland. Scottish Natural Heritage Commissioned Report F99PA08.

Mair, J.M., Moore, C.G., Lyndon, A.R. & Sotheran, I. (2010) Biotope Mapping and Survey of Sullom Voe Special Area of Conservation. A report for Scottish Natural Heritage. Heriot-Watt University, Edinburgh.

Malagoli, D., Casarini, L., Sacchi, S. & Ottaviani, E. (2007). Stress and immune response in the mussel *Mytilus galloprovincialis*. *Fish & Shellfish Immunology*, 23(1), 171-7.

Malone, R.C., Smith, R.D., Maltrud, M.E. & Hecht, M.W. (2003) Eddy-Resolving Ocean Modeling. *Los Alamos Science*, 28, 223-231.

Mariani, S., Peijnenburg, K.T.C.A & Weetman, D. (2012) Independence of neutral and adaptive divergence in a low dispersal marine mollusc. *Marine Ecology Prgress Series*, 446, 173–187.

MSFD (2008) Directive 2008/56/EC of the European Parliament and the Council of 17 June 2008 Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive). <http://ec.europa.eu/environment/water/marine/index_en.htm>.

Matear, R.J. & A.C. Hirst (2003) Long-term changes in dissolved oxygen concentrations in the ocean caused by protracted global warming. *Global Biogeochemical Cycles*, 17, 1125.

Mattoo, O.B., Ivanina, A.V, Ullstad, C., Beniash, E. & Sokolova, I.M. (2013) Interactive effects of elevated temperature and $CO_{(2)}$ levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 164(4), 545-53.

Maxwell, S.L., Venter, O., Jones, K.R. & Watson, J.E. (2015) Integrating human responses to climate change into conservation vulnerability assessments and adaptation planning. *Annals of the New York Academy of Sciences*, 1355(1), 98-116.

McDonagh, B., Tyther, R. & Sheehan, D. (2005) Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and Western blotting: Actin as a target for oxidative stress. *Aquatic Toxicology (Amsterdam, Netherlands)*, 73(3), 315–26.

Met Office & Environment Agency (2016) UK Climate Projections. UKCP09 website: http://ukclimateprojections.metoffice.gov.uk/ Acessed January 2014 - February 2017.

Michaelidis, B., Ouzounis, C., Paleras, A. & Pörtner, H.O. (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovinciallis*. *Marine Ecology Progress Series*, 293, 109–118.

Mies, M. & Sumida, P.Y. (2012) Giant clam aquaculture: a review on induced spawning and larval rearing. *International Journal of Marine Science*, 2(1).

Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G.P. & Marin, M.G. (2007) Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. *Fish & Shellfish Immunology*, 22(1-2), 98–114.

Morris, E. (2015) Defining Annex I biogenic *Modiolus modiolus* reef habitat under the Habitats Directive, JNCC Report 531, ISSN 0963-8901.

Morrison, W.E., Nelson, M.W., Howard, J.F., Teeters, E. J., Hare, J. A. *et al.* (2015) Methodology for assessing the vulnerability of marine fish and shellfish species to a changing climate. NOAA Technical Memorandum NMFS-OSF-3.

Matozzo, V., Chinellato, A., Munari, M., Bressan, M. & Marin, M. G. (2013) Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis*? *Marine Pollution Bulletin*, 72(1), 34–40.

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M. *et al.* (2013) Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), Icelandic scallop (*Chlamys islandica*) and Atlantic cod (*Gadus morhua*): implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology (Amsterdam, Netherlands)*, 127, 21–35.

National Biodiversity Network (NBN) (2016) NBN Atlas website: http://www.nbnatlas.org. Accessed April 2016.

National Oceanic and Atmospheric Administration (NOAA) (2016) NOAA dataset. https://www.nodc.noaa.gov/cgi-bin/OC5/gin-seas-climate/ginregcl.pl. Accessed April 2016.

Navarro, J.M. (1990) Influence of the natural food supply on the physiological energetics and biochemical storage cycles of the horse mussel, *Modiolus modiolus* (Linnaeus). Dissertation, Memorial University of Newfoundland, St. John's.

Navarro, J.M. & Thompson, R. (1996) Physiological energetics of the horse mussel *Modiolus modiolus* in a cold ocean environment. *Marine Ecology Progress Series*, 138, 135–148.

Navarro, J.M., Torres, R., Acuña, K., Duarte, C., Manriquez, P.H. *et al.* (2012) Impact of medium-term exposure to elevated pCO2 levels on the physiological energetics of the mussel *Mytilus chilensis*. *Chemosphere*, 90(3), 1242-1248.

Nestlerode, J.A., Luckenbach, M.W. & O'Beirn, F.X. (2007) Settlement and survival of the oyster *Crassostrea virginica* on created oyster reef habitats in Chesapeake Bay. *Restoration Ecology*, 15, 273-283.

Olbert, A.I., Dabrowski, T., Nash, S. & Hartnett, M. (2012) Regional modelling of the 21st century climate changes in the Irish Sea. *Continental Shelf Research*, 41, 48–60.

Orban, E., Di Lena, G., Nevigato, T., Casini, I., Marzetti, A. *et al.* (2002) Seasonal changes in meat content, condition index and chemical composition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. *Food Chemistry*, 77(1), 57-65.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C. *et al*. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, 437, 681–686.

Osovitz, C.J. & Hofmann, G. (2007) Marine macrophysiology: studying physiological variation across large spatial scales in marine systems. *Comparative Biochemistry and Physiology A*, 147, 821–827.

OSPAR Commission (2009) Background Document for *Modiolus modiolus* beds. OSPAR Biodiversity Series. Prepared on behalf of the Joint Nature Conservation Committee by Rees, E.

Owens, T.G. & King, F.D. (1975) The measurement of respiratory electron-transport-system activity in marine zooplankton. *Marine Biology*, 30(1), 27-36.

Palumbi, S.R. (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics*, 25(1), 547-572.

Palumbi, S.R. (2003) Population genetics, demographic connectivity, and the design of marine reserves. *Ecological Applications*, 13, S146–S158.

Peck, L.S., Clark, M.S., Morley, S.A., Massey, A. & Rossetti, H. (2009) Animal temperature limits and ecological relevance: effects of size, activity and rates of change. *Functional Ecology*, 23(2), 248–256.

Philipp, E.E.R., Wessels, W., Gruber, H., Strahl, J., Wagner, A.E. *et al.* (2012) Gene Expression and Physiological Changes of Different Populations of the Long-Lived Bivalve *Arctica islandica* under Low Oxygen Conditions. *PloS One*, 7(9), e44621.

Pianka, E. R. (1970) On r- and K-Selection. *The American Naturalist*, 104(940), 592–597.

Pörtner, H.O. (2001) Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*, 88(4), 137–146.

Pörtner, H.O. (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 132(4), 739–61.

Pörtner, H.O. (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series*, 373, 203–217.

Pörtner, H.O. (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *Journal of Experimental Biology*, 213(6), 881–93.

Pörtner, H.O. (2012) Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Marine Ecology Progress Series*, 470, 273-290.

Pörtner, H.O. & Farrell, A.P. (2008) Physiology and climate change. *Science*, 322(5902), 690–692.

Pörtner, H.O., Langenbuch, M. & Reipschlager, A. (2004) Biological Impact of Elevated Ocean CO2 Concentrations: Lessons from Animal Physiology and Earth History. *Journal of Oceanography*, 60(4), 705–718.

Pörtner, H.O., Langenbuch, M. & Michaelidis, B. (2005) Synergistic effects of temperature extremes, hypoxia, and increases in CO2 on marine animals: from Earth history to global change. *Journal of Geophysical Research: Oceans*, 110(C9).

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155(2), 945-959.

Qiagen (2006) DNeasy Blood and Tissue Handbook. www.QIAGEN.com July 2006.

Rabalais, N.N., Diaz, R.J., Levin, L.A., Turner, R.E., Gilbert, D. *et al.* (2010) Dynamics and distribution of natural and human-caused hypoxia. *Biogeosciences*, 7(2), 585-619.

Racila, D. & Bickenbach, J.R. (2009) Are epidermal stem cells unique with respect to aging? *Aging*, 1(8), 746–50.

Ragnarsson, S.A. & Burgos, J.M. (2012) Separating the effects of a habitat modifier, *Modiolus modiolus*, and substrate properties on the associated megafauna. *Journal of Sea Research*, 72, 55-63.

Rahmstorf, S. (1995) Multiple convection patterns and thermohaline flow in an idealized OGCM. *Journal of Climate*, 8, 3028–3039.

Rastrick, S.P.S. & Whiteley, N.M. (2013) Influence of natural thermal gradients on whole animal rates of protein synthesis in marine gammarid amphipods. *PloS One*, 8(3), e60050.

Read, K.R. & Cumming, K.B. (1967) Thermal tolerance of the bivalve molluscs *Modiolus modiolus* L., *Mytilus edulis* L. and *Brachidontes demissus* dillwyn. *Comparative Biochemistry and Physiology*, 22(1), 149-155.

Reece, K.S, Ribeiro, W.L., Gaffney, P.M., Carnegie, R.B. & Allen, S.K. (2004) Microsatellite marker development and analysis in the eastern oyster (*Crassostrea virginica*): confirmation of null alleles and non-mendelian segregation ratios. *Journal of Heredity*, 95, 346–352

Rees, E.I.S. (2009) Assessment of *Modiolus modiolus* beds in the OSPAR area. Prepared on behalf of Joint Nature Conservation Committee.

Rees, E.I.S., Sanderson, W.G., Mackie, A.S.Y. & Holt, R.H.F. (2008) Small-scale variation within a *Modiolus modiolus* (Mollusca: Bivalvia) reef in the Irish Sea. III. Crevice, sediment infauna and epifauna from targeted cores. *Journal of the Marine Biological Association of the UK*, 88, 151-156.

Regoli, F., Principato, G., Bertoli, E., Nigro, M. & Orlando, E. (1997) Biochemical characterization of the antioxidant system in the scallop *Adamussium colbecki*, a sentinel organism for monitoring the Antarctic environment. *Polar Biology*, 17(3), 251–258.

Reid, P.C., Edwards, M., McQuatters-Gollop, A., Beaugrand, G., Bresnan, E. *et al.* (2010) Charting Progress 2: Healthy and Biological Diverse Seas Feeder Report, UKMMAS (2010) Charting Progress 2 (2010): 69.

Riisgård, H.U. & Larsen, P.S. (1995) Filter-feeding in marine macro-invertebrates: pump characteristics, modelling and energy cost. *Biological Reviews*, 70(1), 67–106.

Riisgård, H.U., Kittner, C. & Seerup, D.F. (2003) Regulation of opening state and filtration rate in filter-feeding bivalves (*Cardium edule, Mytilus edulis, Mya arenaria*) in response to low algal concentration. *Journal of Experimental Marine Biology and Ecology*, 284(1), 105-127.

Roberts, D., Davies, C., Mitchell, A., Moore, H., Picton, B. *et al.* (2004) Strangford Lough Ecological Change Investigation (SLECI). Report to Environment and Heritage Service by the Queen's University, Belfast.

Roberts, D., Allcock, A.L., Fariñas-Franco, J.M., Gorman, E., Maggs, C. *et al.* (2011) Modiolus Restoration Research Project: Final Report and Recommendations. Queen's University Belfast, Belfast.

Robins, P.E., Neill, S.P., Gimenez, L., Jenkins, S.R. & Malham, S.K. (2013) Physical and biological controls on larval dispersal and connectivity in a highly energetic shelf sea. *Limnological Oceanography*, 58(2), 1–21.

Roe, J.B. & Daily, R.E. (1966) Determination of glycogen with the anthrone reagent. *Analytical Biochemistry*, 5, 245-50.

Rost, B., Zondervan, I. & Wolf-Gladrow, D. (2008) Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions. *Marine Ecology Progress Series*, 373, 227-237.

Rouse, S., Jones, M.E.S. & Porter, J. S. (2014) Spatial and temporal patterns of bryozoan distribution and diversity in the Scottish sea regions. *Marine Ecology* 35(1), 85-102.

Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K. *et al*. (2004) The oceanic sink for anthropogenic CO_2. *Science*, 305(367): 367-71.

Sánchez-Paz, A., Garcia-Carreño, F., Hernández-López, J., Muhlia-Almazán, A. & Yepiz-Plascencia, G. (2007) Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). *Journal of Experimental Marine Biology and Ecology*, 340, 184e93.

Sanderson, W.G., Holt, R.H.F., Kay, L., Ramsay, K., Perrins, J. *et al.* (2008) Small-scale variation within a *Modiolus modiolus* (Mollusca: Bivalvia) reef in the Irish Sea. II. Epifauna recorded by divers and cameras. *Journal of the Marine Biological Association of the United Kingdom*, 88, 143–149.

Sanford, E. & Kelly, M.W. (2011) Local Adaptation in Marine Invertebrates. *Annual Review of Marine Science*, *3*(1), 509–535.

Scheiner, S. (1993) Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics*, *24*.

Schulte, P.M., Healy, T.M. & Fangue, N.A. (2011) Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integrative and Comparative Biology*, 51(5), 691-702.

Schunter, C., Carreras-Carbonell, J., Macpherson, E., Tintoré, J., Vidal-Vijande, E. *et al.* (2011) Matching genetics with oceanography: directional gene flow in a Mediterranean fish species. *Molecular Ecology*, *20*(24), 5167–5181.

Seed, R. & Brown, R.A. (1977) A comparison of the reproductive cycles of *Modiolus modiolus* (L.), *Cerastoderma* (=Cardium) *edule* (L.), and *Mytilus edulis* L. in Strangford Lough, Northern Ireland. *Oecologia*, *30*(2), 173–188.

Selkoe, K.A. & Toonen, R.J. (2011) Marine connectivity: A new look at pelagic larval duration and genetic metrics of dispersal. *Marine Ecology Progress Series*, *436*, 291–305.

Sheehan, D. & McDonagh, B. (2008) Oxidative stress and bivalves: a proteomic approach. *Invertebrate Survival Journal*, 5, 110-123.

Singh, N.P., McCoy, M.T., Tice, R.R., & Schneider, E.L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175(1), 184–91

Slesser, G. & Turrell, W.R. (2013) Annual Cycles of Physical, Chemical and Biological Parameters in Scottish Waters. Scottish Marine and Freshwater Science Vol 4 No 1. Edinburgh: Scottish Government, 17pp.

Smedley, M. (2015) Impacts of thermal and hypoxic stress on the horse mussel (*Modiolus modiolus*). MSc Dissertation, Heriot-Watt University, Edinburgh.

Smolders, R., Bervoets, L., De Coen, W. & Blust, R. (2004) Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environmental Pollution*, 129, 99-112.

Sokolova, I.M. (2013) Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology*, 53(4), 597-608.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G. & Sukhotin, A.A. (2012) Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research*, 79, 1–15.

Solomon, S., Qin, D. Manning, M., Marquis, M., Averyt, K. *et al*. [Eds.] (2007) *Climate Change 2007: The Physical Science Basis*. Cambridge University Press, Cambridge, U.K. 996 pp.

Somero, G.N. (2002) Thermal Physiology and Vertical Zonation of Intertidal Animals: Optima, Limits, and Costs of Living. *Integrative and Comparative Biology*, 42, 780–789.

Somero, G.N. (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”. *Journal of Experimental Biology,* 213(6), 912–20.

Somero, G.N., Bernard, C., Bohr, C. & Krogh, A. (2011) Comparative physiology: a “crystal ball” for predicting consequences of global change. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 301, R1–R14.

Sorte, C.J. & Hofmann, G.E. (2004) Changes in latitudes, changes in aptitudes: *Nucella canaliculata* (Mollusca: Gastropoda) is more stressed at its range edge. *Marine Ecology Progress Series*, 274, 263-268.

Southward, A.J., Hawkins, S.J. & Burrows, M.T. (1995) Seventy years' observations of changes in distribution and abundance of zooplankton and intertidal organisms in the western English Channel in relation to rising sea temperature. *Journal of Thermal Biology*, 20(1–2), 127–155.

Spicer, J.I. & Gaston, K.J. (1999) Physiological diversity and its ecological implications. Oxford, UK: Blackwell Science.

Stillman, J.H. & Tagmount, A. (2009) Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, *Petrolisthes cinctipes*. *Molecular Ecology*, 18(20), 4206-4226.

Storey, K.B. & Tanino, K.K. (2012) Temperature adaptation in a changing climate: Nature at Risk (Vol. 3). CABI.

Taban, I.C., Bechmann, R.K., Torgrimsen, S., Baussant, T. & Sanni, S. (2004) Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. *Marine Environmental Research*, 58, 701–705.

Talmage, S. C., & Gobler, C. J. (2010) Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(40), 17246–51.

Thompson, R.J., Ratcliffe, N.A. & Bayne, B.L. (1974) Effects of starvation on structure and function in the digestive gland of the mussel (*Mytilus edulis* L.). *Journal of the Marine Biological Association of the UK*, 54(3), 699-712.

Thomsen, J. & Melzner, F. (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. *Marine Biology*, 157(12), 2667–2676.

Thomsen, J., Casties, I., Pansch, C., Körtzinger, A. & Melzner, F. (2012) Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global Change Biology*, 19(4), 017-1027.

Tillin, H.M., Hull, S.C. & Tyler-Walters, H. (2010) Development of a sensitivity matrix (pressures-MCZ/MPA features). Report to the Department of the Environment, Food and Rural Affairs from ABPmer, Southampton and the Marine Life Information Network (MarLIN) Plymouth: Marine Biological Association of the UK.145 pp.

Tomanek, L. (2008) The importance of physiological limits in determining biogeographical range shifts due to global climate change: the heat shock response. *Physiological and Biochemical Zoology*, 81, 709–17

Tomanek, L. (2011) Environmental Proteomics: Changes in the Proteome of Marine Organisms in Response to Environmental Stress, Pollutants, Infection, Symbiosis, and Development. *Annual Review of Marine Science*, 3(1), 373–399.

Tomanek, L. (2012) Environmental proteomics of the mussel *Mytilus*: implications for tolerance to stress and change in limits of biogeographic ranges in response to climate change. *Integrative and Comparative Biology*, *52*(5), 648–64.

Tomanek, L. & Somero, G.N. (1999) Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus Tegula) from different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology*, 202(21), 2925–2936.

Torres, G., Giménez, L. & Anger, K. (2007) Effects of osmotic stress on crustacean larval growth and protein and lipid levels are related to life-histories: The genus Armases as a model. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology*, 148(2), 209-224.

Trenberth, K.E., Jones, P.D., Ambenje, P., Bojariu, R., Easterling, D. *et al.* (2007) Observations: Surface and Atmospheric Climate Change. In: *Climate Change 2007: The Physical Science Basis*. Contribution of Working Group I to the Fourth

Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

UK BAP (2008) UK Biodiversity Action Plan, Priority Habitat Descriptions: Horse Mussel Beds and Blue mussel beds on sediment. From: UK Biodiversity Action Plan; Priority Habitat Descriptions. BRIG (ed. Ant Maddock) 2008.

UK Marine Monitoring and Assessment Strategy (UKMMAS) (2010) Charting Progress 2: The State of UK Seas. Ocean Processes Feeder Report: Section 3.2: Temperature and Salinity.

Van Hoey, G., Borja, A., Birchenough, S., Buhl-Mortensen, L., Degraer, S. *et al.* (2010) The use of benthic indicators in Europe: From the Water Framework Directive to the Marine Strategy Framework Directive. *Marine Pollution Bulletin*, 60, 2198-2196.

Van Winkle, W. (1968) The effects of season, temperature and salinity on the oxygen consumption of bivalve gill tissue. *Comparative Biochemistry and Physiology - Part A*, 26(1): 69-80.

Vaquer-Sunyer, R. & Duarte, C.M. (2008) Thresholds of hypoxia for marine biodiversity. *Proceedings of the National Academy of Sciences*, 105, 15452-15457.

Viarengo, A., Canesi, L., Pertica, M. & Livingstone, D.R. (1991) Seasonal variations in the antioxidant defense enzymes and lipid peroxidation of the digestive gland of mussels. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 100, 187–190.

Vosloo, D., van Rensburg, L. & Vosloo, A. (2013) Oxidative stress in abalone: The role of temperature, oxygen and L-proline supplementation. *Aquaculture*, 416, 265-71.

Wan, Q.-H., Wu, H., Fujihara, T. & Fang, S.-G. (2004) Which genetic marker for which conservation genetics issue? *Electrophoresis*, 25(14), 2165–76.

Wang, W.X. & Widdows, J. (1993) Metabolic responses of the common mussel *Mytilus edulis* to hypoxia and anoxia. *Marine Ecological Progress Series*, 95, 205–214.

Wang, X., Wang, L., Zhang, H., Ji, Q., Song, L. *et al.* (2012) Immune response and energy metabolism of *Chlamys farreri* under *Vibrio anguillarum* challenge and high temperature exposure. *Fish and Shellfish Immunology*, 33(4), 1016–26.

Weersing, K. & Toonen, R.J. (2009) Population genetics, larval dispersal, and connectivity in marine systems. *Marine Ecology Progress Series*, 393, 1–12.

Weir, B.S. (1996) Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sinauer, Sunderland, MA, USA.

Weir, B.S. & Cockerham, C.C. (1984) Estimating F-Statistics for the Analysis of Population Structure. *Evolution, 3*8, 1358-1370.

Whitehead, A. (2012) Comparative genomics in ecological physiology: toward a more nuanced understanding of acclimation and adaptation. *The Journal of Experimental Biology*, 215(6), 884–91.

Whitehead, A., Roach, J. L., Zhang, S. & Galvez, F. (2011) Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *Proceedings of the National Academy of Sciences*, *108*(15), 6193-6198.

Whiteley, N. (2011) Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series*, 430, 257–271.

Whiteley, N.M. & Mackenzie, C.L. (2016) Physiological responses of marine invertebrates to thermal stress. In: Solan M, Whiteley NM (eds) *Stressors in the marine environment: physiological and ecological responses and societal implications*. Oxford University Press, Oxford, pp 56-72.

Whitlock, M.C. (2008) Evolutionary inference from QST. *Molecular Ecology*, *17*(8), 1885–96.

Widdicombe, S. & Spicer, J.I. (2008) Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *Journal of Experimental Marnie Biology and Ecology* 336, 187-197.

Widdows, J. & Bayne, B.L. (1971) Temperature acclimation of *Mytilus edulis* with reference to its energy budget. *Journal of the Marine Biological Associaton of the UK*, 51(4), 827–843.

Wildish, D., Fader, G.B., Lawton, P. & MacDonald, A. (1998) The acoustic detection and characteristics of sublittoral bivalve reefs in the Bay of Fundy. *Continental Shelf Research*, 18(1), 105–113.

Willmer, P., Stone, G. & Johnston, I. (2009) *Environmental Physiology of Animals*. John Wiley & Sons.

Wittmann, A. C., & Pörtner, H. (2013) Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change*, *3*(9), 1–7.

Woodin, S.A., Hilbish, T.J., Helmuth, B., Jones, S.J. & Wethey, D.S. (2013) Climate change, species distribution models, and physiological performance metrics: Predicting when biogeographic models are likely to fail. *Ecology and Evolution*, 3(10), 3334–3346.

Wright, S. (1978) *Evolution and the genetics of populations. variability within and among natural populations*, vol 4. University of Chicago Press, Chicago.

Yao, C.-L. & Somero, G. N. (2012) The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *Mytilus californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways. *The Journal of Experimental Biology*, 215(24), 4267–77.

Young, B., Byers, E., Gravuer, K., Hall, K., Hammerson, G. & Redder, A. (2011) Guidelines for using the NatureServe climate change vulnerability index. NatureServe, Arlington, Virginia, USA.

Zacharias, M.A. & Gregr, E.J. (2005) Sensitivity and vulnerability in marine environments: an approach to identifying vulnerable marine areas. *Conservation Biology*, 19, 86–97.

Zampoukas, N., Piha, H., Bigagli, E., Hoepffner, N., Hanke, G. & Cardoso, A. C. (2012) *Monitoring for the Marine Strategy Framework Directive: Requirements and Options*. European Commission Joint Research Centre Institute for Environment and Sustainability.

Zandee, D.I., Holwerda, D.A. & De Zwaan, A. (1980) Energy metabolism in bivalves and cephalopods. *Animals and Environmental Fitness*, 1, 185-206.

Zhang, D.X. & Hewitt, G.M. (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, 12, 563-584.

Zielinski, S. & Pörtner, H.O. (1996) Energy metabolism and ATP free-energy change of the intertidal worm, *Sipunculus nudus*, below a critical temperature. *Journal of Comparative Physiology B*, 166, 492–500.

Zollner, N. & Kirsch, K. (1962) Microdetermination of lipids by the sulphophosphovanillin reaction. *Z Ges Exp Med*, 135, 545-561.